Pharmac. Ther. A, Vol. 2, pp. 1–88, 1977. Pergamon Press. Printed in Great Britain

Specialist Subject Editor: SIDNEY PESTKA

INDUCERS AND INDUCTION OF INTERFERONS

PAUL F. TORRENCE

Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, US National Institutes of Health, Bethesda, Maryland 20014, USA

and

ERIK DE CLERCQ

The Rega Institute, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000, Leuven, Belgium

1. ABBREVIATIONS

Abbreviations for nucleic acids conform to the recommendations of the IUPAC–IUB commission on Biochemical Nomenclature [Biochemistry 9, 4022–4027 (1970)] and are as follows: $(A)_n$, poly(adenylic acid); $(U)_n$, poly(uridylic acid); $(I)_n$, poly(inosinic acid); $(C)_n$, poly(cytidylic acid); $(G)_n$, poly(guanylic acid); $(X)_n$, poly(xanthylic acid); $(rT)_n$, poly(ribothymidylic acid); $(br^5U)_n$, poly(5-bromouridylic acid); $(br^5C)_n$, poly(5-bromocytidylic acid); $(m^5C)_n$, poly(5-methylcytidylic acid); $(s^5C)_n$, poly(5-mercaptocytidylic acid); $(s^2C)_n$, poly(2-thiocytidylic acid); $(fl^5U)_n$, poly(5-fluorocytidylic acid); $(ms^2I)_n$, poly(2-methylthioinosinic acid); $(m_2^2G)_n$, poly(N,N-dimethylguanylic acid); $(ac^4C)_n$, poly(N-acetylcytidylic acid); $(c^7I)_n$, poly(7-deazainosinic acid); $(c^7A)_n$, poly(7-deazaadenylic acid); $(c^3A)_n$, poly(3-deazaadenylic acid); poly(L), poly(laurusin monophosphate) or poly(formycin B monophosphate), the polynucleotide derived from the nucleoside 1,6-dihydro-3-β-D-ribofuranosyl-7H-pyrazolo[4,3-d]pyrimidin-7-one; $(dA)_n$, poly(2′-deoxyadenylic acid); $(dT)_n$, poly(2′-deoxythymidylic acid); $(dI)_n$, poly(2′-deoxyinosinic acid); $(dC)_n$, poly(2′-deoxycytidylic acid); $(dG)_n$, poly(2′-deoxyguanylic acid); $(dU)_n$, poly(2′-deoxyuridylic acid); $(Um)_n$, poly(2′-*O*-methyluridylic acid); $(CM)_n$, poly(2′-*O*-methylcytidylic acid); $(Im)_n$, poly(2′-*O*-methylinosinic acid); $(Am)_n$, poly(2′-*O*-methyladenylic acid); $(dUz)_n$, poly(2′-azido-2′-deoxyuridylic acid); $(dCz)_n$, poly(2′-azido-2′-deoxycytidylic acid); $(dUfl)_n$, poly(2′-fluoro-2′-deoxyuridylic acid); $(Ae)_n$, poly(2′-*O*-ethyladenylic acid); $(Ue)_n$, poly(2′-*O*-ethyluridylic acid). Polynucleotides with a phosphorothioate backbone are symbolized by subscript s; thus $(_sC)_n$ is a homopolymer of cytidine-5′-*O*-thiophosphate residues. Random copolymers are denoted by a comma between the two nucleotide units; thus, $(U_4,C_3)_n$ is a random copolymer of uridylic acid and cytidylic acid in a 4 to 3 ratio. Alternating copolymers are symbolized with a hyphen between the two components; thus, $(dA\text{-}dT)_n$ is an alternating sequence ($dA_pdT_pdA_pdT_pdA\ldots$) copolymer of deoxyadenylic acid and deoxythymidylic acid. Non-covalent associations between polynucleotides are indicated by a center dot; $(I)_n\cdot(C)_n$ represents the 1:1 complex of poly(inosinic acid) and poly(cytidylic acid). Other abbreviations are: mRNA, messenger RNA; dsRNA, double-stranded RNA.

2. INTRODUCTION

Nearly 80 years have passed since Paul Ehrlich defined and pioneered the science of chemotherapy; nonetheless, today we remain without any effective chemotherapy of diseases of viral origin. Viruses, more than any other organism, have consistently resisted virtually all attempts at chemical control. This is not to imply that several important successes have not been claimed. Agents that have demonstrated efficacy in the prophylaxis or therapy of viral infections include 5-iodo-2′-deoxyuridine (treat-

ment of herpes keratitis), amantadine (influenza A prophylaxis) and methisazone (N-methylisatin-β-thiosemicarbazone) (prophylaxis of variola major and variola minor). These successes, although encouraging, have been modest indeed, and it is understandable that considerable excitement greeted the discoveries that the antiviral substance, interferon (Isaacs and Lindenmann, 1957), could be induced by synthetic nucleic acids (Field *et al.*, 1967a) and low molecular weight substances (Krueger and Mayer, 1970; Mayer and Krueger, 1970). In retrospect, the interferon mediated antiviral activity of nucleic acid macromolecules was a finding of double impact; not only could nucleic acids provide antiviral protection, but the possibility arose that such macromolecules themselves could be designed as chemotherapeutic agents, thereby providing the opportunity to draw upon the storehouse of knowledge of the organic chemistry and physical chemistry of polynucleotides.

Conceptually, the design of synthetic polynucleotides as potential chemotherapeutic agents is attractive since: (a) such substances are closely related to an organism's DNAs and RNAs which ultimately direct the synthesis of all constituents of life; (b) structural modification at the monomer level will produce modulation of the configurational and conformational parameters of nucleic acids. The basis for this latter statement lies in the fact that a change of a ribose 2'-OH (RNA) group to a 2'-hydrogen (DNA), coupled with the substitution of a methyl group (DNA) for a hydrogen (RNA) in the base uracil, effects dramatic consequences in terms of nucleic acid form and function.

Both semantically and philosophically, the use of synthetic polynucleotides as antiviral agents (or antitumor agents) cannot be regarded as classical chemotherapy in the sense that Ehrlich first defined it (i.e. the use of chemicals to damage the infecting organism without damage to the host) (Albert, 1973). The weight of evidence shows that synthetic polynucleotides exert their antiviral properties through the natural interferon defense system; they do not seem to act either directly on the virus or on the various steps of viral replication. The antitumor properties of nucleic acids are a somewhat different matter. Interferon may or may not be involved in such antitumor properties, but it is clear that other factors, including a direct effect on the metabolism of tumor cells, must be considered. If this latter suggestion is correct, then synthetic nucleic acids could be considered the first example of violation of Ehrlich's thesis that effective chemotherapeutic agents will be of relatively low molecular weight.

Since interferon is responsible for the antiviral properties of at least several polynucleotides, it is fair to ask what is the purpose of developing interferon inducers which are at least one biological step removed from the desired biological response and which possess additional undesirable (toxic) side-effects? The following potential advantages exist for the production of *endogenous* interferon as opposed to administration of *exogenous* human interferon for the prophylaxis or therapy of viral diseases.

(a) Although major advances have been made recently in the large-scale production of human interferon, there is no contesting the fact that each dose is quite expensive ($500/10^7$ units or one dose). Furthermore, the greatest barrier to demonstrating unequivocally the efficacy of interferon in the clinic is supply. Even if interferon demonstrated efficacy for the treatment of a specific viral disease, there seems no doubt that insufficient quantities could be made available for general usage in all but the rarest viral infections.

(b) Probably the most pressing problem in the interferon field today is its purification. Coupled with this is a significant objection to all clinical trials of interferon conducted to date. The interferon preparations which have been used in clinical trials so far may be estimated to contain only 0.1 per cent (or less) interferon. In marked contrast, both low and high molecular weight inducers are pure chemical substances. Although synthetic polynucleotides are heterogeneous in terms of polymer molecular weight, the *chemical identity of the monomeric subunit, and thus of the polymer itself, is unambiguously defined.*

(c) Because synthetic nucleic acids and agents of low molecular weight would

necessarily have a totally different pharmacology (absorption, biological half-life, distribution, degradation, excretion, etc.) than the interferon protein, they could provide an alternative approach in the event that purified interferon proved to have unfavorable pharmacokinetics.

(d) Even if interferon inducers were to prove unsuitable for direct administration to man, they will remain necessary to produce the enormous amounts of interferon needed for an exogenous interferon therapy.

Other more fundamental reasons for the study of interferon induction include: (a) interferon induction provides an opportunity to study one of the few examples of protein induction in an eukaryotic system. These inquiries are of intense interest for the understanding of the regulation of gene expression in higher organisms; (b) interferon induction provides an excellent opportunity to examine the responses of a biological system to defined changes in the chemical constitution of nucleic acids; and (c) as will be outlined in this review, nucleic acids have diverse other biological effects that may or may not be related to interferon induction. Since the interferon induction system is so far the best defined of all the biological responses of nucleic acids, its study may lay down some fundamental considerations that will be applicable to all these systems.

Herein, we will attempt to describe those substances that are known to induce interferon, the mechanism by which such induction occurs, the structural features that govern induction, and, finally, some other biologic responses shown by interferon inducers. This task is considerably simplified due to the earlier published monographs and reviews on interferon and interferon inducers (Colby, 1971; Colby and Morgan, 1971; Tytell and Field, 1972; Kleinschmidt, 1972; Ng and Vilček, 1972; Grossberg, 1972; Rodgers and Merigan, 1972; Field, 1973; De Clercq, 1973, 1974; De Clercq and Stewart, 1973; Finter, 1973; Pitha and Hutchinson, 1977).

3. GENERAL SURVEY OF INTERFERON INDUCERS

Rather schematically, interferon inducers could be divided into six major classes (Table 1), the first being the class of the viruses which are able to induce interferon in a wide variety of cell systems, both *in vitro* (cell cultures) and *in vivo* (animals). Most, if not all, viruses may owe their interferon-inducing capacity to double-stranded RNA, either present in the virion itself or formed during the replicative cycle of the virus. Double-stranded RNAs are among the most potent interferon inducers. They can be of either viral, non-viral or synthetic origin, and are effective in a wide variety of cells both *in vitro* and *in vivo*. The third class of the interferon inducers consists of the bacterial extracts (endotoxins). They are effective only *in vivo* (mice, rabbits, ... and leukocyte cultures), as are the polycarboxylates (class IV), some low-molecular-weight substances (class V) and factors which specifically stimulate T or B lymphocytes (class VI). These different classes of interferon inducers will be discussed in detail. Particular emphasis will be on the synthetic inducers of interferon such as synthetic double-stranded RNAs, as they permit establishment of a structure–function relationship between the inducer and the interferon response it elicits. Synthetic double-stranded RNAs have also proven useful in studies directed at deciphering the mechanism of interferon induction (and 'superinduction') at the cellular level.

TABLE 1. *Major Classes of Interferon Inducers*

Class	Agents	Cell types which respond to interferon induction
I	Viruses	Wide variety of somatic cells
II	Double-stranded RNAs	Wide variety of somatic cells
III	Endotoxins	Lymphoreticular cells
IV	Polycarboxylates	Lymphoreticular cells
V	Low molecular weight compounds	Lymphoreticular cells
VI	T or B Lymphocyte stimulating agents	Lymphocytes

Some interferon inducers have not been accommodated in Table 1 and will not be discussed further in our review. These inducers include microorganisms other than viruses (protozoa, bacteria, rickettsiae, chlamydiae) and a number of cellular extracts (of either bacterial or fungal origin), the interferon inducing capacity of which has been reported some time ago (for review, see De Clercq, 1973). Some of these inducers (e.g. chlamydiae and rickettsiae) may, in analogy with viruses, operate in a wide variety of cells *in vitro* and *in vivo*. Others (e.g. bacteria, protozoa, and bacterial and fungal extracts) may, like endotoxin, operate only in a limited number of cells *in vivo*. As not much progress has been made with these interferon inducers during the last 5 years, they will not be dealt with here.

A new group of interferon inducers has emerged recently: the mycoplasmas. Several strains of mycoplasmas have been shown to induce interferon in cultures of ovine peripheral blood leukocytes (Rinaldo *et al.*, 1973) and in mice (Rinaldo *et al.*, 1974). In mice, peak interferon titers were attained 6 hr after intraperitoneal inoculation of the mycoplasmas, quite similar to what is observed upon injection of (nonreplicating) viral inducers of interferon. Mycoplasmas failed to induce interferon in mouse spleen cells, mouse peritoneal cells and mouse blood leukocyte cultures (Rinaldo *et al.*, 1974); however, some mycoplasma strains induced interferon in human lymphocyte cultures (Cole *et al.*, 1976). Since interferon induced by mycoplasma, like 'classical' virus-induced interferon, retained its antiviral activity upon exposure to pH 2, it could be considered as Type I interferon according to the nomenclature introduced by Youngner and Salvin (1973) (see Section 7 on 'Interferon induction by factors stimulating T and B lymphocytes').

4. LOW-MOLECULAR-WEIGHT INDUCERS OF INTERFERON

The fact that interferon can be induced by low-molecular-weight substances became apparent in studies aimed at delineating the influence of protein synthesis inhibitors on interferon formation in mice (Youngner *et al.*, 1965; Youngner, 1970b). The protein synthesis inhibitors themselves (cycloheximide, acetoxycycloheximide, streptovitacin A, streptimidone and tenuazonic acid) proved effective in stimulating interferon production, and the amounts of interferon produced correlated closely with the extent of inhibition of protein synthesis (Youngner *et al.*, 1965; Youngner, 1970b). Recently, a new glutarimide antibiotic, 9-methylstreptimidone, has been added to the list of glutarimides inducing interferon. More importantly, 9-methylstreptimidone was found to protect mice against a lethal influenza A_2 virus infection, when administered before virus challenge (Saito *et al.*, 1976). Another antibiotic which has been found effective in inducing interferon is kanamycin which elicited a biphasic interferon response in chickens (Lukáš and Hrušková, 1968). This observation has, however, never been confirmed or extended.

The first synthetic low-molecular-weight compound reported to induce interferon was tilorone (dihydrochloride) (Krueger and Mayer, 1970; Mayer and Krueger, 1970). The compound was particularly effective when given orally to mice. The optimal dose amounted to 250 mg/kg, and the serum interferon titers peaked at 18–24 hr after oral administration of tilorone (Mayer and Krueger, 1970; De Clercq and Merigan, 1971b; Stringfellow and Glasgow, 1972b). As a direct result of its interferon inducing ability, tilorone conferred protection against a wide variety of virus infections (reviewed in Table 2). As commonly noted with interferon inducers, tilorone was effective only when administered prophylactically (Krueger and Mayer, 1970). Due to hyporeactivity, tilorone quickly lost its interferon inducing potency upon repeated administration of the inducer (Stringfellow and Glasgow, 1972a). Since the extent of antiviral protection closely parallels the titers of interferon induced by different doses of tilorone (De Clerq and Merigan, 1971b), there is little doubt that the antiviral activity of tilorone is mediated by interferon production. That Giron *et al.* (1972c) obtained protection against MM virus infection with doses of tilorone which did not induce detectable amounts of circulating interferon, should not necessarily be con-

TABLE 2. *Synthetic Low Molecular Weight Compounds which have been shown to Stimulate Interferon Production and to Confer Antiviral Protection*

Compound	Host	Administration route	Interferon induction	Antiviral activity	Leading references
Fluorenone esters Aminoacyl fluorenes Anthraquinone sulfonamides Anthraquinone ethers Fluoranthene esters	Mouse	p.o., s.c.	+	EMC SFV Vaccinia	Sill *et al.*, 1973, 1974 Albrecht *et al.*, 1974a, b Grisar *et al.*, 1974
Fluorenone ethers (e.g. tilorone)	Mouse	p.o., s.c., i.p.	+	EMC VSV SFV HSV Influenza FMDV	Krueger and Mayer, 1970 Mayer and Krueger, 1970 De Clercq and Merigan, 1971b Krueger *et al.*, 1971 Stringfellow and Glasgow, 1972b Richmond and Campbell, 1973 Andrews *et al.*, 1974 Stringfellow *et al.*, 1974
N,N-dioctadecyl-N',N'-bis(2-hydroxyethyl) propanediamine	Mouse	i.p.	+	EMC SFV Vaccinia	Hoffman *et al.*, 1973
Pyrazolo[3,4-b]quinoline derivatives	Mouse	p.o., i.p.	+	EMC SFV Vaccinia HSV	Siminoff *et al.*, 1973 Siminoff, 1975, 1976 Crenshaw *et al.*, 1976 Kern *et al.*, 1976*b*
Acridine derivatives —Acranil, quinacrine	Mouse	p.o., s.c.	+	Vaccinia	Gláz *et al.*, 1973 Gláz and Tálas, 1975
—10-carboxymethyl-9-acridanone	Mouse	p.o., s.c., i.p.	+	SFV Coxsackie WEE HSV	Kramer *et al.*, 1976
Bis-ω-piperidinylacetyl-dibenzofuran	Mouse	p.o.	+	EMC VSV HSV *FMDV*	Richmond and Campbell, 1973 Soehner *et al.*, 1974
Pyrimidine derivatives (e.g. 2-amino-5-bromo-6-methyl-4-pyrimidinol)	Mouse	p.o., i.p.	+	EMC	Nichol *et al.*, 1976

Abbreviations: p.o., perorally; s.c., subcutaneously; i.p., intraperitoneally; EMC, encephalomyocarditis (virus); SFV, Semliki forest virus; VSV, vesicular stomatitis virus; HSV, herpes simplex virus; FMDV, foot-and-mouth disease virus; WEE, Western equine encephalitis (virus).

Note: although basic dyes such as toluidine blue, methylene blue, trypaflavine and acridine orange have been accredited with interferon-inducing properties (Diederich *et al.*, 1972, 1973), they have not been included in the present list of low molecular weight inducers of interferon, since the antiviral principle found in the serum of mice treated with these dyes did not follow the time response curve which is characteristic for interferon production.

sidered as evidence against a direct correlation between interferon induction and antiviral protection, since the assay employed to titrate interferon may not have been sufficiently sensitive to detect low interferon titers.

After the disclosure of tilorone dihydrochloride as an interferon inducer, various other synthetic low-molecular-weight compounds have been described to stimulate interferon production in mice. The active compounds belong to widely different chemical classes: fluorenone esters (Sill *et al.*, 1973), fluorenone ethers (Andrews *et al.*, 1974), aminoacyl fluorenes (Albrecht *et al.*, 1974a), anthraquinone sulfonamides (Grisar *et al.*, 1974), anthraquinone ethers (Sill *et al.*, 1974), fluoranthene esters (Albrecht *et al.*, 1974b), pyrazolo[3,4-b]quinoline derivatives (Crenshaw *et al.*, 1976), acridine derivatives (Glaz *et al.*, 1973; Kramer *et al.*, 1976), propanediamine derivatives (Hoffman *et al.*, 1973), pyrimidine derivatives (Nichol *et al.*, 1976) and a

dibenzofuran derivative (Soehner *et al.*, 1974). The structure of the most representative congeners of these different classes is depicted in Fig. 1. Their ability to induce interferon and resistance to virus infection is reviewed in Table 2. All compounds induced interferon and exhibited antiviral activity in mice, when administered via the oral or parenteral route. Peak interferon titers in mouse sera generally occurred 18–24 hr after drug administration, except for the interferon induced by 2-amino-5-bromo-6-methyl-4-pyrimidinol, which peaked at 6–12 hr (Nichol *et al.*, 1976). Where it was tested, antiviral protection correlated well with interferon production, and interferon production decreased upon repeated administration of the inducer (hyporeactivity) (Siminoff *et al.*, 1973; Kern *et al.*, 1976b).

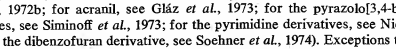
The interferon produced in response to low-molecular-weight interferon inducers is, in analogy with the interferon induced by viruses and double-stranded RNAs, resistant to pH 2.0 (for tilorone, see Mayer and Krueger, 1970 and Stringfellow and Glasgow, 1972b; for acranil, see Gláz *et al.*, 1973; for the pyrazolo[3,4-b]quinoline derivatives, see Siminoff *et al.*, 1973; for the pyrimidine derivatives, see Nichol *et al.*, 1976; for the dibenzofuran derivative, see Soehner *et al.*, 1974). Exceptions to this rule are the interferons induced by 9-methylstreptimidone and N,N-dioctadecyl-N',N'-bis (2-hydroxyethyl) propanediamine. These interferons are readily inactivated upon exposure to pH 2.0 (Saito *et al.*, 1976; Hoffman *et al.*, 1973).

What is most typical of the low-molecular-weight inducers of interferons is: (a) that they are active upon oral administration; (b) that they are particularly active in mice but not in other animal species (including man); and (c) that, despite the lack of structural similarity among the different classes of low-molecular-weight inducers of interferon, there is, within each class, a rather strict limitation of possible chemical modifications or substitutions if the property of interferon induction is to be retained. Due to diversity in chemical structure of the different classes of low-molecular-weight

FLUORENE, FLUORENONE, FLUORANTHENE AND ANTHRAQUINONE DERIVATIVES

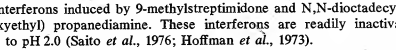
FLUORENONE ESTERS :

e. g. bis [3-(dibutylamino)propyl] fluorenone-2, 7-dicarboxylate dihydrochloride



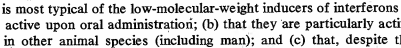
FLUORENONE ETHERS :

e. g. 2, 7-bis [2-(diethylamino)ethoxy] fluorenone dihydrochloride (tilorone dihydrochloride)



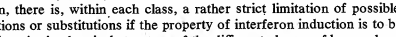
AMINOACYL FLUORENES :

e. g. 2, 7-bis [2-(diethylamino)acetyl] fluorene dihydrochloride



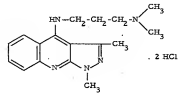
ANTHRAQUINONE SULFONAMIDES :

e. g. bis [3-(dibutylamino)propyl] anthraquinone-2, 6-disulfonamide dihydrochloride



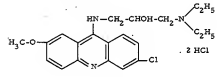
PYRAZOLO [3,4-b] QUINOLINE DERIVATIVES

e.g. 1,3-dimethyl-4-(3-dimethylaminopropylamino)-1 H-pyrazolo [3,4-b] quindine dihydrochloride



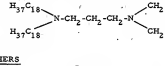
ACRIDINE DERIVATIVES

e.g. 2-methoxy-6-chloro-9-(2-hydroxy-3-diethylaminopropylamino)-acridine dihydrochloride (acranil dihydrochloride)



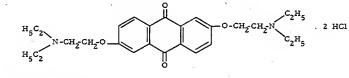
PROPANEDIAMINE DERIVATIVES

e.g. N,N-dioctadecyl-N',N'-bis(2-hydroxyethyl)propanediamine



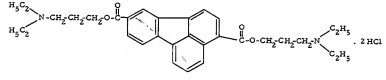
ANTHRAQUINONE ETHERS

e.g. 2,6-bis [2-(diethylamino)ethoxy] anthraquinone dihydrochloride



FLUORANTHENE ESTERS

e.g. bis [3-(diethylamino)propyl] fluoranthene-3,9-dicarboxylate dihydrochloride



PYRIMIDINE DERIVATIVES

e.g. 2-amino-5-bromo-6-methyl-4-pyrimidinol

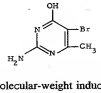


FIG. 1. Low-molecular-weight inducers of interferon.

inducers of interferon, it is at the present time impossible to formulate structural requirements that are common for all low-molecular-weight inducers. The structural requirements which hold for one class of inducers cannot be extrapolated to other classes of low-molecular-weight inducers of interferon.

As alluded to above, low-molecular-weight inducers of interferon are very restrictive as far as the host or host cells in which they initiate an interferon response. Their activity is exquisite in mice. In other animals (rabbits, chicks. . . .) or cell cultures, they are either inactive or very poorly active. Some interferon inducing activity was noted with tilorone in human lymphocyte cell cultures, but this activity could be considered as the direct consequence of a toxic alteration of the cells (Dennis *et al.*, 1972). Although inactive by itself, tilorone proved capable of augmenting the interferon-inducing potential of mixtures of $(I)_n \cdot (C)_n$ and DEAE (diethylaminoethyl)-dextran in mouse L cells (Groelke *et al.*, 1975).

In man, tilorone proved entirely inefficient in inducing interferon in the blood (upon oral administration) and in tears (upon administration as eye drops) (Kaufman *et al.*, 1971). Kaufman *et al.* (1971) concluded from their studies that tilorone was neither safe nor effective for use in humans. The only other low-molecular-weight compound which has so far been submitted to clinical trials is N,N-dioctadecyl-N′,N′-bis-(2-hydroxyethyl)-propanediamine. When administered topically (as nose drops) to volunteers with rhinovirus infection, the propanediamine derivative resulted in the development of interferon in nasal secretions and a mild but discernible reduction of the severity of illness (Gatmaitan *et al.*, 1973; Douglas and Betts, 1974). With a microdispersed preparation containing drug particles less than 2 μm in diameter, even greater amounts of interferon were detected in the nasal washings, and, concomitantly, virus shedding and symptoms of rhinovirus disease were markedly reduced (Panusarn *et al.*, 1974). However, Douglas *et al.* (1975) failed to extend the latter observations to volunteers infected with influenza A virus. Hence, the clinical usefulness of low-molecular-weight inducers of interferon cannot be fully appraised at the present time.

The restricted number of cell systems which respond to the low-molecular-weight inducers of interferon raises the question as to the sort of cells which account for the interferon produced in the intact animal. The primary target cell of the pyrazolo[3,4-b]quinoline derivatives may well be the 'fixed' macrophage (macrophage which adheres to a plastic surface) of the reticuloendothelial tissues (spleen, liver,. . .) (Siminoff, 1975, 1976). Lymphocytes, including T lymphocytes, do not seem to be involved in the interferon response of the host to low-molecular-weight compounds, since tilorone retains its full interferon-inducing potential in both ALS (antilymphocyte serum)-treated mice and congenitally athymic (nude) mice (which lack T lymphocytes) (Stringfellow and Glasgow, 1972b; Gibson *et al.*, 1976).

5. INTERFERON INDUCTION BY POLYCARBOXYLATES

The ability of synthetic anionic polymers to induce interferon formation in mice was first noted for a series of maleic anhydride copolymers (prototype: maleic anhydride divinylether copolymer, also called 'pyran' copolymer) (Merigan, 1967; Merigan and Regelson, 1967; Merigan and Finkelstein, 1968) and later extended to polyacrylic acid (PAA) (De Somer *et al.*, 1968b) and a series of chlorite-oxidized oxypolysaccharides (prototype: chlorite-oxidized oxyamylose or COAM) (Claes *et al.*, 1970). The primary chemical structure of pyran copolymer, PAA and COAM is depicted in Fig. 2. Prominent features of their structure are a high molecular weight (exceeding 10,000), a stable backbone and the presence of negative charges (carboxyl groups) (as reviewed by De Clercq, 1973). PAA and pyran copolymer possess a –C–C–C–C–C–backbone. This makes them poorly degradable within the organism. COAM has a –C–C–O–C–O–backbone, and is, therefore, more readily degraded within the organism than PAA or pyran copolymer. Anionic character is the most stringent requirement for macromolecules to be active as interferon inducers. Uncharged macromolecules (e.g. polyacrylamide) and polycations (e.g. DEAE-dextran) are inactive in inducing interferon. The anionic character may be provided by carboxyl or any other negatively charged group, e.g. sulfate groups, as present in polyvinylsulfate (Came *et al.*, 1969), or phosphate groups, as present in dextran

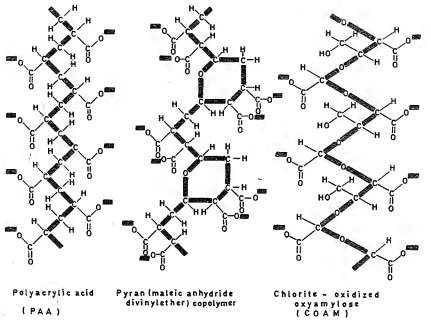


FIG. 2. Structures of three polycarboxylate interferon inducers drawn to emphasize the long-chain backbone of negative charges arranged in a regular and dense sequence.

phosphate (Suzuki *et al.*, 1975). Although the interferon inducing ability of polyanions is inherently linked to the anionic character of the polymer, it is not known how the density, strength and spatial orientation of the anionic groups modulate the general behavior of the polymer as interferon inducer.

In addition to their interferon inducing capacity, PAA, pyran copolymer and COAM also confer resistance to a number of viral infections, as summarized in Table 3. As with the low-molecular-weight inducers of interferon, interferon induction by polycarboxylates has almost exclusively been shown in mice. For both the low-molecular-weight inducers and the polycarboxylates, serum interferon titers reach their peak value at 18–24 hr after drug administration. While low-molecular-weight inducers are exquisitely effective upon oral administration, polycarboxylates should be administered by the intraperitoneal route to yield greatest efficacy. Whether the interferon produced in response to polycarboxylates should be considered as acid-labile or acid-stable is open to debate. Thus, Merigan (1967) reported mouse serum interferon induced by pyran copolymer to be inactivated by acid treatment (pH 2), whereas De Somer *et al.* (1968a) found mouse serum interferon induced by PAA perfectly resistant to pH 2 treatment.

Polycarboxylates imparted greatest protection against virus infection when administered prophylactically, e.g., 24 hr before virus challenge. If treatment was delayed until several days after virus challenge, protective activity rapidly decreased (De Clercq and De Somer, 1968b; Billiau *et al.*, 1971b). Administration of the polycarboxylate installed a state of hyporeactivity (tolerance) during which a new injection of the drug failed to trigger the host's interferon response (De Somer *et al.*, 1968b; Merigan and Finkelstein, 1968). Hyporeactivity to interferon induction was paralleled by a hyporeactivity to antiviral protection (De Clercq *et al.*, 1970d). Whereas the prophylactic effects of other interferon inducers (double-stranded RNAs, low-molecular-weight compounds) on virus infections wane within a few days, single doses of PAA, pyran copolymer and COAM afforded a prolonged protection against pox tail lesion formation (induced by intravenous vaccinia virus challenge) and mortality (induced by intraperitoneal mengovirus inoculation). This protection lasted at least 1–2 months (De Clercq and De Somer, 1968b; Merigan and Finkelstein, 1968; Billiau *et al.*, 1970a). The long-term protection offered by polycarboxylates is ob-

TABLE 3. *Synthetic Polycarboxylates which have been shown to Stimulate Interferon Production and to Confer Antiviral Protection*

Compound	Host	Administration route	Interferon induction	Antiviral activity	Leading references
Polyacrylic acid (PAA)	Mouse	i.p.	+	VSV	De Somer *et al.*, 1968b
				SFV	De Clercq and De Somer, 1968a, b, 1969
				Vaccinia	Billiau *et al.*, 1970a
				HSV	Leunen *et al.*, 1971
				FMDV	De Clercq and Luczak, 1976b
	Mouse	i.m., i.v.	ND	Vaccinia	De Clercq and De Somer, 1969
	Rabbit	i.p.	—	Vaccinia	De Clercq and De Somer, 1973a
Pyran (maleic anhydride divinyl ether) copolymer	Mouse	i.p.	+	EMC	Merigan, 1967
				VSV	Merigan and Regelson, 1967
				MM	Merigan and Finkelstein, 1968
				Mengo	De Clercq and Merigan, 1969b
				Vaccinia	De Clercq *et al.*, 1970d
				HSV	Pindak *et al.*, 1971
				FMDV	Richmond, 1971a
					Richmond and Campbell, 1972
					Morahan *et al.*, 1972b
					Morahan and McCord, 1975
					McCord *et al.*, 1976
Chlorite-oxidized oxyamylose (COAM)	Mouse	i.p.	+	VSV	Claes *et al.*, 1970
				SFV	Billiau *et al.*, 1970a,
				Mengo	1971a, b, 1972a
				Influenza	Leunen *et al.*, 1971
				Vaccinia	De Clercq and De Somer,
				HSV	1971b
				FMDV	De Clercq and Luczak, 1976a

Abbreviations: i.p., intraperitoneally; i.v., intravenously; i.m., intramuscularly; EMC, encephalomyocarditis (virus); SFV, Semliki forest virus; VSV, vesicular stomatitis virus; HSV, herpes simplex virus; FMDV, foot-and-mouth disease virus.

viously related to the lack of biodegradability and deposition of the polyanion in the reticuloendothelial cells. As reviewed previously (De Clercq, 1973), polycarboxylates not only exhibit antiviral activity in animal model infections, they also inhibit tumor growth and afford protection against bacterial, fungal and protozoal infections. Are all these effects mediated by interferon production? There are some observations which suggest that the antiviral effects of polycarboxylates could be mediated by interferon production: (a) the compounds are most effective when given shortly (24 hr) before virus challenge, that is, at the time circulating interferon titers reach their peak value (cf. references in Table 3); (b) implantation of methyl groups on the backbone of PAA results in a significant reduction in interferon inducing capacity, and, concomitantly, annihilates the inhibitory effect of PAA on vaccinia virus-induced pox lesions (De Somer *et al.*, 1968b; De Clercq and De Somer, 1968b; Billiau *et al.*, 1972a); (c) analogs of either PAA, COAM or pyran copolymer, which are not equipped with a sufficient number of carboxyl groups, fail to induce interferon (De Somer *et al.*, 1968b; Merigan and Finkelstein, 1968; Claes *et al.*, 1970), and, in addition, fail to protect mice against viral and bacterial infections (De Clercq and De Somer, 1968b; Remington and Merigan, 1970); and (d) as alluded to above, hyporeactivity to interferon production is paralleled by hyporeactivity to antiviral protection (De Clercq *et al.*, 1970d).

There are, however, various observations which seem to refute the likelihood that the antiviral and other biological activities of polycarboxylates are mediated by interferon production: (a) in many instances the antiviral (or antibacterial) activity persisted for several weeks, thus much longer than interferon could be detected in the bloodstream (Merigan and Finkelstein, 1968; De Clercq and De Somer, 1968b, 1969; Billiau *et al.*, 1970a; Remington and Merigan, 1970); (b) although PAA failed to stimulate interferon production in rabbits, it protected rabbits against vaccinia virus-induced skin lesions (De Clercq and De Somer, 1973a); (c) COAM, when given

intraperitoneally to mice, increased the titer of serum interferon subsequently induced by $(I)_n \cdot (C)_n$ (De Clercq *et al.*, 1972a; Levy *et al.*, 1973); however, this increased interferon output was not reflected by an increased protection against rabies infection (Harmon *et al.*, 1974); (d) when both mengovirus and COAM were administered by the intraperitoneal route, the antiviral protection conferred by the polycarboxylate correlated with a retention ('trapping') of the virus in the peritoneal cavity (Billiau *et al.*, 1971a). The protective activity observed in the latter conditions was attributed to an activation of the peritoneal macrophages. This activation might result in an increased phagocytosis of virus, increased destruction of virus, or both.

Activation of macrophages could also be invoked to explain the protective effects of pyran copolymer on Rauscher leukemia virus, polyoma virus and herpes simplex virus infections in immunosuppressed mice (Hirsch *et al.*, 1972; Morahan and McCord, 1975). The antitumor activity of pyran copolymer may also reside with activated macrophages, since pyran copolymer potentiates the specific cytotoxicity of macrophages for target tumor cells (Kaplan *et al.*, 1974; Schultz *et al.*, 1976). Activation of macrophages might eventually emerge as the common denominator underlying the antiviral activity and other biological activities of polycarboxylates. Such a unifying hypothesis should not necessarily be interpreted as evidence against the role of interferon in the biological activities of polycarboxylates, since activated macrophages may at least partly act through the elaboration of an intermediary substance such as interferon.

6. INTERFERON INDUCTION BY BACTERIAL COMPONENTS (ENDOTOXINS)

Endotoxins have been the first agents of non-viral origin found to stimulate interferon production (Ho, 1964; Stinebring and Youngner, 1964). The most characteristic difference between the patterns of interferon induction by bacterial endotoxins (and bacteria) on the one hand, and viruses on the other, is that with bacteria and bacterial endotoxins maximum interferon production is obtained at 2 hr, whereas virus-induced interferon attains its peak titer at about 12 hr after intravenous injection of the inducer to mice (Stinebring and Youngner, 1964). *Brucella abortus* constitutes a notable exception to this rule, since *Brucella abortus*, much like viruses, elicits a late interferon response (Stinebring and Youngner, 1964; Youngner and Stinebring, 1964; De Somer *et al.*, 1970).

Interferon induction studies with bacterial endotoxins have been most regularly performed in mice; however, the interferon inducing activity of endotoxin is not limited to this animal species. Unlike polycarboxylates (e.g. polyacrylic acid) and low-molecular-weight compounds (e.g. tilorone dihydrochloride), endotoxins are quite effective interferon inducers in rabbits (Ho, 1964; Ho and Kono, 1965b; Ho *et al.*, 1973; Ho *et al.*, 1976). *Brucella abortus*, however, is not (De Clercq and De Somer, 1973a). To evaluate its interferon inducing properties, endotoxin is generally administered parenterally, e.g. intravenously or intraperitoneally. However, Youngner (1972) and Borecký *et al.* (1973) ascertained that endotoxin, when given by the oral route to mice, also induces significant amounts of interferon. Endotoxin is one of the most powerful producers of hyporeactivity: injection of endotoxin leads to a period of tolerance during which the interferon response to a second dose of either endotoxin or other interferon inducers, e.g. viruses or $(I)_n \cdot (C)_n$, is greatly reduced (Ho *et al.*, 1965; Youngner and Stinebring, 1965; Ho *et al.*, 1970).

In contrast with virus-induced interferon which would originate through *de novo* synthesis, endotoxin was originally considered to stimulate the release of preformed interferon. This distinction was based primarily on the different effects of antimetabolites on the induction of interferon by endotoxin and virus in animals. For example, cycloheximide was reported to inhibit interferon induction by virus while potentiating interferon induction by endotoxin (Youngner *et al.*, 1965). Later on, De Clercq and Merigan (1970) indicated that the data obtained with cycloheximide did not

provide evidence for two distinct mechanisms of interferon induction. They found that, depending on the time cycloheximide was administered, both virus- and endotoxin-induced interferon could be either suppressed or augmented. De Clercq and Merigan (1970) concluded that both virus- and endotoxin-induced interferon require *de novo* protein synthesis. This contention was later confirmed by Ho *et al.* (1973).

In terms of chemical structure, endotoxins are poorly defined. They correspond to the lipopolysaccharide component of the cell wall of gram-negative bacteria. As the lipopolysaccharides of different gram-negative bacteria do not constitute a single entity, their interferon-inducing potency may vary considerably. Moreover, some bacteria may contain more than one interferon-inducing principle. The active interferon-inducing components which have been extracted from bacteria are summarized in Table 4. The interferon inducing capacity of *Escherichia coli* is generally associated with its lipopolysaccharide content. Yet, Morahan and Grossberg (1970) succeeded in isolating a protein from *E. coli* which induced 'interferon-like' resistance to virus infection in chick embryos. *E. coli* mutants deficient in cell-wall lipopolysaccharide offered antiviral resistance equivalent to that provided by the wild-type *E. coli* (Morahan and Grossberg, 1970). The interferon inducing capacity of *Pseudomonas aeruginosa* and *Bordetella pertussis* also appears to reside in more than one component: lipopolysaccharide and (glyco)protein (Kojima *et al.*, 1971, 1973). For *Klebsiella pneumoniae*, the interferon inducing activity seems to reside in the polysaccharide moiety of the capsular antigen (Kato *et al.*, 1975). Protein or lipid would play only a minor

TABLE 4. *Active Interferon-inducing Components Present in Bacteria*

Bacteria	Active moiety	Host	Administration route	Leading references
Escherichia coli	Lipopolysaccharide	Mouse	i.v.	Stinebring and Youngner, 1964 Youngner and Stinebring, 1965, 1966 Youngner, 1970a Ito *et al.*, 1971, 1973
		Rabbit	i.v.	Ho, 1964 Ho and Kono, 1965b Ho *et al.*, 1965, 1970, 1973, 1976
		Mouse	i.v., i.p., s.c., p.o.	Borecky *et al.*, 1973
		Rat	i.v.	De Somer and Billiau, 1966
		Chick embryo	i.a.	Morahan and Grossberg, 1970
Salmonella typhimurium	Lipopolysaccharide (Lipid A)	Mouse	i.v.	Youngner and Feingold, 1967 Feingold *et al.*, 1968, 1970 Youngner *et al.*, 1973
		Mouse	p.o.	Youngner, 1972
Hemophilus influenzae	Capsular polyribophosphate?	Mouse	i.p.	De Clercq and Merigan, 1969c
Aerobacter	Not identified	Mouse	i.v.	Youngner, 1970a
Pseudomonas aeruginosa	1°) Lipopolysaccharide 2°) Protein	Rabbit	i.v.	Kojima *et al.*, 1971
Bordetella pertussis	1°) Lipopolysaccharide 2°) Glycoprotein	Rabbit	i.v.	Kojima *et al.*, 1973
Serratia	Lipopolysaccharide	Mouse	i.v.	Galabov and Galabov, 1973
Klebsilla pneumoniae	Capsular polysaccharide	Mouse	i.v.	Kato *et al.*, 1975
Brucella abortus	1°) Lipopolysaccharide	Mouse	i.v.	Billiau *et al.*, 1970b Keleti *et al.*, 1974 Bousquet *et al.*, 1976
	2°) Structural component	Mouse	i.v., i.p.	De Somer *et al.*, 1970 Billiau *et al.*, 1970b Youngner *et al.*, 1974 Kern *et al.*, 1976a Feingold *et al.*, 1976

Abbreviations: i.v., intravenously; i.p., intraperitoneally; s.c., subcutaneously; p.o., perorally; i.a., intra-allantoically.

role, if any, in the interferon-inducing effect of this capsular polysaccharide. For *Salmonella typhimurium*, however, the interferon-inducing property of the lipopolysaccharide uniquely resides in the lipid A portion of the molecule (Youngner *et al.*, 1973).

For *Brucella abortus*, at least two active principles have been detected. The first principle corresponds to lipopolysaccharide, and, although it is much less effective than the lipopolysaccharide isolated from *Salmonella typhimurium*, it produces a typical endotoxin-type of interferon response with peak serum interferon levels occurring 2 hr after intravenous injection to mice (Keleti *et al.*, 1974). This principle cannot be held responsible for the rather protracted interferon response (with peak serum interferon titers at 6–8 hr noted upon the injection of intact brucellae. The active component responsible for the virus-type of interferon response of *Brucella abortus* has not yet been isolated. Heating at 80°C, exposure to alkali (NaOH 0.1 N) and extraction with aqueous ether rendered brucellae nonviable but did not abolish their interferon inducing capacity (De Somer *et al.*, 1970; Billiau *et al.*, 1970b; Youngner *et al.*, 1974). Treatment with phenol, trichloracetic acid (TCA) or sodium dodecyl sulphate (SDS) and mechanical disruption destroyed the interferon inducing ability of brucellae (De Somer *et al.*, 1970; Billiau *et al.*, 1970b). It was suggested, therefore, that the interferon inducing activity of *Brucella abortus* depended on the maintenance of the structural integrity of the cell (Youngner *et al.*, 1974). While an ethanol extract as well as the residue of the ethanol extraction did not prove very efficient in inducing interferon, the original interferon inducing activity of the intact brucellae could be restored, at least partially, when the extract and the residue were recombined (De Somer *et al.*, 1970). Quite similar results have recently been reported with *Brucella abortus* extracted with a 2:1 (v/v) mixture of chloroform and methanol (C–M) (Feingold *et al.*, 1976). The C–M extracted brucellae, while inactive themselves, regained full activity when recombined with the C–M extract from either *Brucella* or *E. coli.* Neither extracted *E. coli* reconstituted with *Brucella* extract nor the extracts alone had any interferon inducing activity. This suggests that the activity of *Brucella* is dependent upon at least two separable components, one of which is C–M soluble and the other C–M insoluble. The soluble component is common to at least two species of bacteria, *Brucella abortus* and *E. coli.* The insoluble component, on the other hand, seems to be unique to *Brucella.* The soluble component may be identified as a lipid, e.g. phosphatidylglycerol or phosphatidylethanolamine, since these phospholipids can substitute in part for the material extracted from *Brucella* or *E. coli* (Feingold *et al.*, 1976). Little information is presently available concerning the unique, nonextractable component of *Brucella.* It may require the structural integrity of the cell rather than involve a single molecule (Feingold *et al.*, 1976).

Only a few of the bacterial extracts listed in Table 4 were studied for their ability to confer resistance to virus infection in experimental animals. Protective activity was noted with the capsular polysaccharide of *Klebsiella pneumoniae* in mice infected with the pneumonitis virus of mice (Ginsberg and Horsfall, 1951) as well as with ether-extracted *Brucella abortus* in mice infected with Semliki forest virus (SFV), encephalomyocarditis (EMC) virus and herpes simplex virus (HSV) (Youngner *et al.*, 1974; Kern *et al.*, 1976a). The antiviral activity of the bacterial extracts may be associated not only with interferon production but with a general increase of the host's resistance, e.g. through activation of cells of the reticuloendothelial system (see Section 5 on interferon induction by polycarboxylates).

Like polycarboxylates and low-molecular-weight inducers of interferon, endotoxins stimulate interferon production in animals but not in conventional cell cultures. After injection of endotoxin into the animal, interferon is produced primarily in liver, lung, spleen and thymus (Ho *et al.*, 1973), as monitored by *in vitro* interferon production with tissue slices removed from rabbits which received an intravenous injection of *E. coli* endotoxin 15 min before they were sacrificed. When explanted *in vitro*, both spleen cells and peritoneal cells produce significant amounts of interferon in response to endotoxin (Kobayashi *et al.*, 1969; Galabov and Galabov, 1973; Borecký *et al.*,

1973). Adherent spleen cells and adherent peritoneal cells produce significantly more interferon than nonadherent cells (Borecký *et al.*, 1973; Ho *et al.*, 1976). These observations suggest that the 'fixed' macrophages are responsible for the interferon produced by spleen and peritoneal cells in response to endotoxin, at least as far as the 'early' interferon is concerned (i.e. the interferon produced within 24 hr). This 'early' interferon is heat-labile (inactivated when exposed to 56° for 30 min). Mouse spleen cells also produce a 'late' interferon 24–48 hr after exposure to endotoxin (Ho *et al.*, 1976). This 'late' interferon is heat-stable (not inactivated when exposed to 56° for 60 min) and may originate from the B lymphocytes (Ho *et al.*, 1976). The finding that thymus tissue removed from endotoxin-treated animals produces interferon when incubated *in vitro* suggests that T lymphocytes may also participate in interferon induction by endotoxin (Ho *et al.*, 1973). Thus, endotoxin appears to act upon a fairly large range of cells (macrophages, T lymphocytes and B lymphocytes). *In vivo* (intact animal), two or more of these cell types may function synergistically to produce interferon in response to endotoxin.

The latter viewpoint is corroborated by Ito *et al.* (1973). They found that splenectomized mice, when treated with endotoxin some time after splenectomy, produced little or no interferon (Ito *et al.*, 1971). However, the interferon producing ability of these mice was restored by intraperitoneal injection, prior to the endotoxin stimulus, of not only syngenic mouse but also xenogeneic rat spleen cells. The interferon produced in the rat-to-mouse chimera exhibited the species specificity of mouse interferon. To examine which cell type of the spleen was responsible for the restoration of interferon production, rat spleen cells were separated into 'glass-adherent' cells (macrophages) and 'nonadherent' cells (lymphocytes). Upon transfer of these fractions to splenectomized mice, only the 'glass-adherent' cells were found to restore the interferon response to endotoxin. From these observations, Ito *et al.* (1973) concluded that the *in vivo* induction of interferon by endotoxin may be achieved by a two-step mechanism. The first step or 'endotoxin-reactive' step would involve the interaction of endotoxin with the splenic macrophages. The second step or 'interferon-producing' step might occur in the lymphocytes of the spleen or any other lymphoid organ. These cells would only produce interferon after they have been 'informed' by the endotoxin-reactive cells to do so.

7. INTERFERON INDUCTION BY FACTORS STIMULATING T AND B LYMPHOCYTES

In 1965, Wheelock demonstrated the presence of an interferon-like substance in the supernatants of human leukocyte cultures stimulated with the nonspecific mitogen, phytohemagglutinin (PHA). The interferon produced in these cultures shared many physical, chemical and biological properties with the 'classical' virus-induced interferon, except stability at pH 2.0. Subsequently, interferon was found in the supernatant fluids of human and mouse leukocytes stimulated with other mitogens such as pokeweed and concanavalin A (Friedman and Cooper, 1967; Wallen *et al.*, 1973; Stobo *et al.*, 1974). That leukocyte cultures, derived from individuals who had been immunized with some antigens (e.g. tuberculin, tetanus toxoid and diphtheria toxoid), released interferon upon renewed contact with the respective antigen, was first demonstrated by Green *et al.* (1969). These studies have later been confirmed and extended to various other antigens, including viral antigens such as vaccinia and herpes simplex virus (Epstein *et al.*, 1972a; Rasmussen *et al.*, 1974; Valle *et al.*, 1975a, b; Haahr *et al.*, 1976). Interferon production through immune recognition also operates *in vivo*, as pointed to by the appearance of interferon in the bloodstream of BCG-infected mice which were challenged some time after BCG infection with old tuberculin (OT) or purified protein derivative (PPD) of tuberculin (Stinebring and Absher, 1970; Salvin *et al.*, 1973; Youngner and Salvin, 1973). A review of the various specific and nonspecific mitogens which are capable of stimulating interferon production by immunocompetent cells is presented in Table 5.

TABLE 5. *Interferon Induction by Immune Recognition*

Inducer	Cell system	Interferon sensitive to pH 2.0	Leading references
1°) *Mitogens*			
Phytohemagglutinin (PHA)	Human blood lymphocytes	+	Wheelock, 1965; Friedman and Cooper, 1967; Green *et al.*, 1969; Epstein *et al.*, 1971, 1974; Haber *et al.*, 1972; Epstein and Cline, 1974; Valle *et al.*, 1975a, b
	Human tonsil lymphocytes	+	Klimpel *et al.*, 1975
	Swine blood leukocytes	+	Richmond, 1969
	Mouse spleen (T) lymphocytes	?	Wallen *et al.*, 1973; Stobo *et al.*, 1974
Concanavalin A (Con A)	Mouse spleen (T) lymphocytes	?	Wallen *et al.*, 1974; Stobo *et al.*, 1974
Pokeweed mitogen (PWM)	Human blood lymphocytes	–	Friedman and Cooper, 1967
	Human tonsil lymphocytes	+	Klimpel *et al.*, 1975
	Mouse spleen (B) lymphocytes	?	Wallen *et al.*, 1973
2°) *Mixed lymphocyte cultures*	Human tonsil lymphocytes	+	Klimpel *et al.*, 1975
	Mouse spleen cells	–	Gifford *et al.*, 1971
3°) *Antilymphocyte antibody*	Human blood lymphocytes	+	Falcoff *et al.*, 1972a, b; Falcoff, 1972
4°) *Specific antigens*			
Purified protein derivative (PPD) (OT: old tuberculin)	Human blood lymphocytes	+	Green *et al.*, 1969; Epstein *et al.*, 1972b
	Bovine blood lymphocytes	?	Babiuk and Rouse, 1976
	Mouse spleen (T) lymphocytes	?	Wallen *et al.*, 1973
	Mouse peritoneal cells	?	Milstone and Waksman, 1970
	Rabbit blood lymphocytes	?	Bartfeld and Vlček, 1975
	Intact mouse (sensitized with BCG)	+	Stinebring and Absher, 1970; Salvin *et al.*, 1973; Youngner and Salvin, 1973; Salvin *et al.*, 1974, 1975
Tetanus toxoid	Human blood lymphocytes	?	Green *et al.*, 1969
Diphtheria toxoid	Human blood lymphocytes	?	Green *et al.*, 1969
Streptolysin-O	Human blood lymphocytes	–	Friedman and Cooper, 1967
Vaccinia virus	Human blood lymphocytes	+	Epstein *et al.*, 1972a
Herpes simplex virus (HSV)	Human blood lymphocytes	+	Rasmussen *et al.*, 1974; Valle *et al.*, 1975a, b; Haahr *et al.*, 1976
Infectious bovine rhinotracheitis virus (IBR)	Bovine blood lymphocytes	?	Babiuk and Rouse, 1976
Newcastle disease virus (NDV)	Mouse peritoneal cells	±	Yamada *et al.*, 1970; Azuma *et al.*, 1970a, b; Azuma, 1972
	Intact mouse (immunized with NDV)	–	Lo and Treagan, 1974
Chikungunya virus	Mouse peritoneal cells	–	Glasgow, 1966
Shope fibroma virus	Rabbit peritoneal cells	–	Pathak and Tompkins, 1974
L-cells	Mouse peritoneal cells	±	Borecký *et al.*, 1971
Endotoxin	Mouse peritoneal cells	?	Borecký *et al.*, 1968

+: denotes interferon is destroyed at pH 2.0; –: indicates that it is stable at pH 2.0; ?: means that the sensitivity of interferon to pH 2.0 was not determined, whereas ± means that the interferon was partly resistant to pH 2.0.

The immune-specific interferon may originate from either T or B lymphocytes, but intimate contact with macrophages is required for maximum interferon production (as reviewed by Epstein, 1976). With human lymphocyte cultures (stimulated by phytohemagglutinin or pokeweed mitogen), T cell interferon production can be assessed at 3 days, whereas B cell interferon production takes 5–7 days (Epstein *et al.*, 1974). Measurement of mitogen-stimulated interferon production at 3 days can be considered as an effective tool to monitor the effector competence of human T lymphocytes; hence, a depressed T lymphocyte interferon production was recorded

for patients with chronic lymphocytic leukemia (Epstein and Cline, 1974) and patients with selective IgA deficiency (Epstein and Ammann, 1974).

Both T and B lymphocyte interferon production is augmented in the presence of macrophages (Epstein *et al.*, 1971, 1972b, 1974; Rasmussen *et al.*, 1974; Valle *et al.*, 1975b; Haahr *et al.*, 1976). The mechanism by which macrophages augment interferon production by lymphocytes is not clearly understood. Most likely, the macrophage promotes the delivery of the mitogen to the lymphocyte in a form that optimizes the interferon triggering process (Epstein, 1976). Macrophage–lymphocyte interaction holds not only for human lymphocyte cultures; the presence of macrophages is also required for bovine and murine lymphocytes to achieve maximum interferon production (Babiuk and Rouse, 1976; Milstone and Waksman, 1970).

In mice which have been immunized with BCG, the maximum interferon titers are obtained 4–6 hr after intravenous injection of PPD (Stinebring and Absher, 1970). The time at which interferon produced by lymphocyte cultures attains its peak value can vary considerably from one system to another: (a) within 24 hr in mouse peritoneal cell cultures (which were derived from tuberculin-sensitized or L cell-immunized animals) exposed *in vitro* to either PPD (Milstone and Waksman, 1970) or L cells (Borecky *et al.*, 1971); also within 24 hr in blood lymphocytes from PPD-sensitive rabbits, when exposed to PPD (Bartfeld and Vilček, 1975); (b) at 24–48 hr in sensitized bovine blood lymphocytes, when exposed to infectious bovine rhinotracheitis (IBR) virus (Babiuk and Rouse, 1976); (c) at 3–4 days for a mixture of genetically dissimilar mouse spleen lymphocytes (Gifford *et al.*, 1971) and for human blood lymphocytes exposed to phytohemagglutinin (Epstein *et al.*, 1971); and (d) at 7–8 days for sensitized human blood lymphocytes, when exposed to PPD (Epstein *et al.*, 1972b). Differences in the kinetics of immune-specific interferon production may depend on a number of factors such as the immune status of the host and the nature of the mitogenic stimulus; thus, with human lymphocytes, interferon produced in response to specific antigens (e.g. PPD) peaks later than interferon produced in response to nonspecific mitogens (e.g. phytohemagglutinin).

Lymphocytes respond to both specific and nonspecific antigens with the production of interferon, as well as with proliferation (blast transformation) and the production of a series of 'mediators of cellular immunity' (also called 'lymphokines'), such as MIF (migration inhibitory factor) and LT (lymphotoxin). Even *in vivo* (BCG-infected mice), production of interferon production (in response to tuberculin) is accompanied by the appearance of various other factors (e.g. MIF and factors which inhibit the growth of microorganisms and retard the proliferation of bone marrow cells) (Salvin *et al.*, 1974, 1975).

In vitro, both the production of lymphokines (including interferon) and blast transformation largely depend on the presence of macrophages (Epstein, 1976). Although interferon production is generally associated with blastogenesis and lymphokine production, quite often there is a dissociation in time between peak interferon production and maximum blast transformation. In addition, several authors have stated that the extent of blast transformation does not correlate with the amount of interferon produced (Green *et al.*, 1969; Epstein *et al.*, 1971; Babiuk and Rouse, 1976). Wallen *et al.* (1973) even succeeded in separating (by discontinuous density gradient centrifugation on bovine serum albumin) the spleen cells which responded to the blastogenic (DNA synthesis-stimulating) effects of PHA and Con A from the spleen cells which produced interferon in reponse to PHA and Con A. Other authors failed to establish a direct correlation between the relative amounts of interferon and MIF or LT produced by either rabbit blood lymphocytes or human tonsil lymphocytes (Bartfeld and Vilček, 1975; Klimpel *et al.*, 1975).

In many instances, the interferon produced in lymphocyte cultures stimulated by specific antigens or nonspecific mitogens was shown to be T cell dependent: e.g. with mouse spleen lymphocytes stimulated by phytohemagglutinin, concanavalin A or PPD (Wallen *et al.*, 1973; Stobo *et al.*, 1974), with bovine blood lymphocytes stimulated by infectious bovine rhinotracheitis (IBR) virus (Babiuk and Rouse, 1976) and with

human blood lymphocytes stimulated by herpes simplex virus (HSV) antigen (Valle *et al.*, 1975a). Treatment of mouse spleen lymphocytes with anti-theta serum plus complement (to kill the T cells) obliterated the interferon response to phytohemagglutinin, concanavalin A and PPD (but not to pokeweed), while treatment with anti-K chain antibody plus complement (to kill the B cells) had no effect on the interferon response to phytohemagglutinin and concanavalin A (Wallen *et al.*, 1973; Stobo *et al.*, 1974). That T cells also participate in the immune-specific induction of interferon in bovine and human lymphocytes, when exposed to either IBR or HSV antigen, was ascertained by using lymphocytes highly enriched in T cells through chromatography on nylon wool columns (Valle *et al.*, 1975a; Babiuk and Rouse, 1976). These experiments suggest that the immune-specific interferon triggered by HSV, IBR, PHA, Con A and PPD originates from T lymphocytes or a subpopulation of T lymphocytes. At the very least, they indicate that T lymphocytes provide the antigenic-specific step for 'immune interferon' production.

There are a few additional observations which may throw some light on the mechanism underlying the immune-specific interferon production by sensitized lymphocytes. These observations concern the enhancement of both (Newcastle disease) virus- and endotoxin-induced interferon production by antibody (Azuma *et al.*, 1970b; Borecký *et al.*, 1968). That antigen-antibody complexes are capable of stimulating the production of interferon and may even induce more interferon than the antigen alone, could be explained by assuming that when complexed to cytophilic antibody (Azuma, 1973), the antigen is processed more readily by the antigen-receptive cells.

A number of criteria can be used to distinguish the 'immune interferon', induced by nonspecific mitogens (e.g. phytohemagglutinin) and specific antigens (e.g. PPD) in immunocompetent cells from the 'classical interferon' induced by viruses and double-stranded RNAs in a much broader range of somatic cells. The term "immune interferon' was originally suggested by Falcoff (1972). It was later designated as Type II interferon by Youngner and Salvin (1973). Type I interferon would correspond to the 'classical interferon' produced by such stimuli as endotoxin, $(I)_n$·$(C)_n$ and Newcastle disease virus. It should be stressed that the terms Type I and Type II interferons were reserved only for the interferons produced in mice and mouse cell cultures (Youngner and Salvin, 1973). Youngner and Salvin (1973) recognized four major differences between Type I interferon and Type II interferon: (1) Type I interferon is stable at pH 2.0, Type II interferon is not; (2) Type II interferon is stable at 56°, Type I interferon is not; (3) Type I interferon is active on primary guinea pig kidney cells, Type II interferon is not; and (4) Type II interferon is not neutralized by antibodies raised against Type I interferon.

These criteria cannot dogmatically be applied to distinguish between the classical and immune interferons of other species (human, rabbit, bovine, . . .).

Criterion (1) is undoubtedly the most stringent of all four. As reviewed in Table 5, the majority of the interferons induced by either nonspecific mitogens (e.g. phytohemagglutinin) or specific antigens (e.g. PPD) have been found sensitive to pH 2.0. Notable exceptions are the interferons produced in mixed mouse spleen cell cultures (Gifford *et al.*, 1971), and in human blood lymphocyte cultures exposed to either pokeweed mitogen or streptolysin-O (Friedman and Cooper, 1967). That the immune-specific interferons induced by some viruses (Newcastle disease virus: Lo and Treagan, 1974; Chikungunya virus: Glasgow, 1966; Shope fibroma virus: Pathak and Tompkins, 1974) proved resistant to pH 2.0 treatment should be interpreted with caution. These interferons were treated at pH 2.0 to inactivate residual virus. The remaining activity may at least partly be accounted for by classical interferon.

Criterion (2) cannot be extrapolated to human interferon. The interferon induced by Newcastle disease virus and herpes simplex virus in normal (non-sensitized) human lymphocytes (thus, classical Type I interferon) is, unlike mouse Type I interferon, stable at 56° (Falcoff, 1972; Haahr *et al.*, 1976). *Vice versa*, the immune (Type II) interferon produced in human lymphocyte cultures in response to phytohemagglutinin

or herpes simplex antigen is quite heat-labile (Valle *et al.*, 1975b). In analogy with mouse Type II interferon, it should have been stable at 56°.

Criterion (3) allows mouse Type I interferon, but not mouse II interferon, to be effective in heterologous cells. Type I interferons of other species, particularly human interferon, cross the species barrier quite often. For example, human leukocyte interferon and human fibroblast interferon, two classical (Type I) interferons, were found to exert a pronounced antiviral activity on rabbit, cat, bovine and porcine cells: human fibroblast interferon was even more active on rabbit cells than on human cells (Desmijter *et al.*, 1968), and human leukocyte interferon was more active on cat cells (Desmijter and Stewart, 1976) and on bovine cells (Gresser *et al.*, 1974) than on human cells. Therefore, the observation that the immune-specific interferon produced by human lymphocyte cultures in response to vaccinia antigen did not confer antiviral protection in feline cells (Epstein *et al.*, 1972a), if confirmed and extended to other immune-specific interferons of human origin, may support the contention that the immune (Type II) interferons are more species specific in their antiviral activity than the classical (Type I) interferons.

Criterion (4) points to the antigenic diversity of mouse Type I and Type II interferon. This criterion seems to hold for human interferon as well. Anti-human interferon antiserum, raised against a classical Type I (leukocyte) interferon, was shown to neutralize human Type I (fibroblast) interferon, yet failed to neutralize human Type II interferon (induced by phytohemagglutinin or herpes simplex antigen) (Valle *et al.*, 1975b).

8. INTERFERON INDUCTION BY VIRUSES

As recently reviewed by Burke (1973), nearly all major virus groups are able to trigger an interferon response. Very schematically, these virus groups could be divided into three classes: (a) viruses containing a double-stranded DNA genome; (b) viruses containing a double-stranded RNA genome; and (c) viruses containing a single-stranded RNA genome.

8.1. DOUBLE-STRANDED DNA VIRUSES

A variety of DNA viruses, including members of the papova-, herpeto-, adeno- and poxviridae as well as T-even coliphages, has been shown to stimulate interferon production; however, the exact nature of the active principle in these DNA viruses is still a matter of conjecture. Kleinschmidt *et al.* (1970) attributed the interferon inducing capacity of T4 coliphage to the DNA component of the phage. Since the extracted DNA failed to induce interferon, Kleinschmidt *et al.* postulated that only the encapsulated DNA, as present inside the phage, possessed the conformation required for triggering the interferon response. It is noteworthy that Kleinschmidt *et al.* measured interferon production in the mouse, and that this system is suitable to demonstrate interferon production with inducers of widely different nature, including endotoxins and low-molecular-weight inducers. In fact, the interferon produced in response to T4 coliphage peaked at 18 hr, which is reminiscent of the interferon response to low-molecular-weight interferon inducers.

Nearly all other DNA viruses were tested for their ability to induce interferon in chick embryo cells. According to Colby and Duesberg (1969), the interferon inducing capacity of vaccinia virus in chick cells would reside in a double-stranded RNA intermediate made during the multiplication cycle of the virus. However, Bakay and Burke (1972) failed to demonstrate any correlation between interferon formation and double-stranded RNA synthesis. They found that with vaccinia virus, double-stranded RNA, but not interferon, was formed, and that with adenovirus, interferon, but not double-stranded RNA, was formed. In other studies (Pusztai *et al.*, 1969), the penton antigen was thought to be responsible for the induction of interferon by adenovirus in chick cells. Studies with temperature-sensitive mutants of adenovirus (Ustacelebi and

Williams, 1972) suggest that some virus function, as yet unidentified, is necessary for interferon production. For avian adenovirus CELO, the interferon inducing capacity does not appear associated with DNA synthesis or virus replication (Markovits and Coppey, 1972). Chick cells have also been employed to monitor interferon induction by polyoma virus (Ustacelebi and Williams, 1973). Complete polyoma virus particles were required for interferon induction. Empty shells failed to induce interferon. Characteristically, SV 40, a papovavirus related to polyoma, failed to induce interferon in chick cells under conditions where polyoma did so (Ustacelebi and Williams, 1973).

8.2. Double-stranded RNA Viruses

In view of the superior interferon inducing activity of synthetic double-stranded RNAs such as $(I)_n \cdot (C)_n$, it is not unreasonable to attribute the interferon inducing potency of double-stranded RNA viruses to their RNA content. Double-stranded RNA isolated from a variety of double-stranded RNA viruses of either bacterial, animal, fungal, plant or insect origin have all been found active in inducing interferon (Table 6). The same applies to the replicative form of some coliphages as well as mammalian viruses (e.g. mengo, influenza and vaccinia virus) (Table 6).

In general, the free double-stranded RNA is a more active interferon inducer than the intact viral particle, and the interferon produced in response to the free RNA peaks earlier than the interferon obtained with the intact virions (see e.g. Tytell *et al.*,

TABLE 6. *Natural Double-stranded RNA which have been Reported to Possess Significant Activity as Interferon Inducers*

Origin of double-stranded RNA	Activity *in vitro*	Activity *in vivo*	Leading references
Reovirus type 3	+	+	Tytell *et al.*, 1967
Mengo virus (replicative form)	+	+	Falcoff and Falcoff, 1970 Falcoff *et al.*, 1973
Influenza virus (replicative form)	+	ND	Colby and Duesberg, 1969
Vaccinia virus (replicative form)	+	ND	Colby and Duesberg, 1969
MS 2 coliphage (replicative form)	+	+	Field *et al.*, 1967a
MU 9 (mutant of MS 2 coliphage) (replicative form)	+	+	Nemes *et al.*, 1969
f1 coliphage (replicative form)	+	+	Nemes *et al.*, 1969 Colby *et al.*, 1971
f2 coliphage (replicative form)	+	+	Doskočil *et al.*, 1971 Gajdošová *et al.*, 1973 Táborský *et al.*, 1974
Pseudomonas phaseolicola Φ6 phage	ND	+	Kleinschmidt *et al.*, 1973, 1974
Cytoplasmic polyhedrosis virus	+	+	Nemes *et al.*, 1969
Rice dwarf virus	+	+	Nemes *et al.*, 1969 Takehara and Suzuki, 1973
Penicillium funiculosum mycophage	+	+	Lampson *et al.*, 1967 Banks *et al.*, 1968 Nemes *et al.*, 1969
Penicillium stoloniferum mycophage	+	+	Kleinschmidt *et al.*, 1968 Banks *et al.*, 1968 Planterose *et al.*, 1970
Penicillium chrysogenum mycophage	+	+	Buck *et al.*, 1971 Edy *et al.*, 1974 De Clercq and Torrence, 1977
Penicillium cyaneo-fulvum mycophage	ND	+	Banks *et al.*, 1969
Aspergillus foetidus mycophage	ND	+	Banks *et al.*, 1970

Note 1: Nemes *et al.* (1969) erroneously referred to a DNA–RNA hybrid as the active principle in f1 coliphage. The interferon inducing capacity of this phage actually resides with double-stranded RNA (Colby *et al.*, 1971).

Note 2: Natural dsRNAs that induce interferon need not be of viral origin. De Maeyer *et al.* (1971) and Kimball and Duesberg (1971) found that dsRNA, isolated from normal apparently uninfected rat liver cells, was able to trigger the production of interferon. In addition, certain strains of *Saccharomyces cerevisiae* carry an endogenous dsRNA plasmid which gives these cells (killers) the ability of secrete a protein that is toxic only to cells that do not carry the plasmid (Wickner, 1976). This 'killer' yeast dsRNA induces interferon in mouse L cells (Bevan *et al.*, 1973).

1967; Planterose *et al.*, 1970; Buck *et al.*, 1971). The slower action of the virus probably reflects the slow release of double-stranded RNA from the virus particles.

Consistent with the hypothesis that the double-stranded RNA of the inoculum virus is responsible for the induction of interferon by double-stranded RNA viruses, are the observations of Long and Burke (1971) and Dubovi and Akers (1972). These authors found reovirus type 3 and Colorado tick fever virus (a member of the orbivirus group) capable of inducing interferon in chick cells or L cells under conditions which did not permit virus replication or virus-specific RNA synthesis. Under conditions which permitted virus replication, however, Gauntt (1973) found no interferon induction with reovirus type 3 in L cells. In a different strain of L cells, Lai and Joklik (1973) effectively showed interferon induction with reovirus type 3, but were unable to correlate the induction of interferon with the double-stranded RNA content of the input material, at least for the virus which produced a lytic infection cycle. For the latter, a very late event in virus replication was implicated in interferon induction. However, Lai and Joklik (1973) did not exclude the possibility that the ability of ultraviolet-irradiated reovirus to induce interferon resided with the double-stranded RNA of the input virions. Taken together, the data of Long and Burke (1971), Dubovi and Akers (1972), Gauntt (1973) and Lai and Joklik (1973) could be interpreted to mean that the double-stranded RNA of the inoculum virus functions as interferon inducer only in temperate conditions, when no lytic infection is generated.

8.3. SINGLE-STRANDED RNA VIRUSES

Guided by the superiority of double-stranded RNA's as interferon inducers, one could argue that single-stranded RNA viruses owe their interferon inducing ability to the double-stranded RNA replicative form made during the multiplication cycle of the single-stranded RNA virus. Even under conditions in which no viral RNA synthesis occurs (as will be discussed below), the interferon induction by single-stranded RNA viruses may be accounted for by traces of double-stranded RNA. Such double-stranded RNA contaminants have been demonstrated experimentally in unpurified concentrates of Newcastle disease virus, Sindbis virus and Semliki Forest virus (Field *et al.*, 1972a).

That the interferon inducing capacity of mengo virus resides with its replicative intermediate and replicative form (which represents the double-stranded portion of the replicative intermediate) has been unequivocally shown by Falcoff and Falcoff (1969, 1970). There are, however, several situations in which single-stranded viruses have been shown not to replicate, not to produce progeny, yet to stimulate the production of interferon. These conditions have been most circumstantially documented for Newcastle disease virus, Semliki Forest virus and Sindbis virus (Table 7). In all conditions listed (e.g. inactivation of the virus by ultraviolet irradiation or heat, or infection of the cells with temperature-sensitive mutants at non-permissive temperatures), virus replication is minimal or non-existent. That interferon was produced in such conditions indicated that the formation of a fully developed replicative intermediate is not a prerequisite for interferon induction. From the studies reviewed in Table 7, it appears as though interferon induction depends on a low threshold level of viral RNA synthesis occurring early in infection, or, where even this limited viral RNA synthesis could be excluded, interferon induction may directly depend on a component of the input virions (e.g. single-stranded RNA).

That interferon may be produced under conditions where single-stranded RNA viruses do not replicate or produce progeny should not necessarily be regarded as evidence against synthesis of limited amounts of double-stranded viral RNA: e.g. ultraviolet-irradiated Newcastle disease virus still retains part of its ability to synthesize RNA after it has lost its infectivity and, concomitantly, the irradiated virus has the capacity to induce interferon (Clavell and Bratt, 1971). With large doses of ultraviolet irradiation, RNA synthesizing capacity and interferon inducing capacity are lost in parallel. These findings cast doubts on the hypothesis that the single-stranded

TABLE 7. *Mechanism of Interferon Induction by Single-stranded RNA Viruses*

Virus	Cell	Conditions	Interferon induction attributed to	References
Mengo	L929	No restrictions	Replicative intermediate (replicative form)	Falcoff and Falcoff, 1969, 1970
Newcastle disease	Chick embryo	Ultraviolet-irradiated virus	Input single-stranded RNA	Gandhi and Burke, 1970
				Gandhi *et al.*, 1970
			Limited viral RNA synthesis (early in infection)	Huppert *et al.*, 1969
				Clavell and Bratt, 1971
				Meager and Burke, 1972
Newcastle disease	L(CCL-1)	Presence of actinomycin D	Input single-stranded RNA	Dianzani *et al.*, 1970, 1974
Chikungunya	Rat embryo			
Semliki forest	Chick embryo	Virus inactivated by heat	Input single-stranded RNA	Goorha and Gifford, 1970, 1971
		Temperature-sensitive mutants	Input single-stranded RNA (at high virus multiplicity)	Lomniczi and Burke, 1970
			Viral RNA synthesis (at low virus multiplicity)	
Sindbis	Chick embryo	Temperature-sensitive mutants	Viral process other than RNA synthesis	Lockart *et al.*, 1968
			Limited viral RNA synthesis (early in infection)	Atkins *et al.*, 1974
				Atkins and Lancashire, 1976

RNA of the input virions acts as the inducer of interferon. As in the case of non-irradiated virus, the RNA polymerase present in the ultraviolet-irradiated Newcastle disease virus particles may still function and transcribe (part of the) viral RNA genome. The resulting base-paired RNA product, whatever length it may attain, would then serve as inducer of interferon. Whether such a limited base-paired RNA intermediate also accounts for the interferon response with viruses other than Newcastle disease virus, e.g. with temperature-sensitive mutants of Sindbis and Semliki Forest virus at nonpermissive temperatures, remains to be established.

9. DO VIRUSES AND DOUBLE-STRANDED RNAs INDUCE INTERFERON THROUGH THE SAME MECHANISM?

Irrespective of the role played by a double-stranded RNA intermediate in the interferon induction process of viruses, the question may be raised whether viruses, as exemplified by Newcastle disease virus, and double-stranded RNAs, as exemplified by $(I)_n \cdot (C)_n$, stimulate the same or different interferon genes and whether the mechanisms controlling their expression differ from one inducer to another.

There are a number of differences in the induction of interferon by $(I)_n \cdot (C)_n$ and Newcastle disease virus:

(a) Exposure of (rabbit or human) cell cultures to $(I)_n \cdot (C)_n$ leads to an early interferon response which peaks about 4 hr after induction and rapidly ceases thereafter; however, inoculation of the same cells with Newcastle disease virus results in a later and more protracted interferon production, peaking 10–15 hr after inoculation (Mozes and Vilcek, 1975).

(b) Metabolic inhibitors exert a differential effect on the induction of interferon by either $(I)_n \cdot (C)_n$ or Newcastle disease virus (Finkelstein *et al.*, 1968; Tan *et al.*, 1970). The induction of interferon by Newcastle disease virus is quite susceptible to inhibition by agents which suppress RNA or protein synthesis. The action of these agents on interferon induction by $(I)_n \cdot (C)_n$ is more complex: depending on the dosage and timing of the treatment with the inhibitor, it may cause either suppression or stimulation of the interferon response.

(c) Ultraviolet irradiation of the cells prior to their exposure to Newcastle disease virus results in a dose-dependent decrease of interferon production. In contrast, interferon production in cells exposed to $(I)_n \cdot (C)_n$ is markedly enhanced upon ul-

traviolet irradiation of the cells (Mozes and Vilček, 1974, 1975). Both DEAE-dextran treatment and priming of the cells with interferon prior to induction further potentiate the enhancing effect of ultraviolet irradiation on the interferon induction by $(I)_n \cdot (C)_n$ in human fibroblast cells (Lindner-Frimmel, 1974; Mozes *et al.*, 1974).

(d) The plant alkaloid camptothecin inhibits interferon induction by Newcastle disease virus and Sindbis virus in chick and/or human cells. It has no effect on interferon production induced by $(I)_n \cdot (C)_n$ in chick and human cells (Atherton and Burke, 1975).

As suggested by Vilček *et al.* (1975), the differences between the characteristics of the $(I)_n \cdot (C)_n$ and Newcastle disease virus-induced interferon responses could be explained by assuming that either: (1) the $(I)_n \cdot (C)_n$ and virus-induced interferons represent different gene products; or (2) both interferons originate from the same structural gene(s) but are expressed through different control mechanisms. If the interferons induced by $(I)_n \cdot (C)_n$ and Newcastle disease virus indeed represent identical gene products, one may expect the two interferons to be neutralized by antibody raised against $(I)_n \cdot (C)_n$-induced interferon. This proved to be the case. Rabbit antiserum, prepared against interferon produced in human fibroblast cells with $(I)_n \cdot (C)_n$, neutralized the activity of both $(I)_n \cdot (C)_n$ and Newcastle disease virus-induced interferon (Havell *et al.*, 1975a). The cross-reactivity between anti-interferon antiserum [raised with $(I)_n \cdot (C)_n$-induced interferon] and virus-induced interferon indicates substantial antigenic homology between the two interferons but does not necessarily imply that their primary structures are identical. In fact, Jankowski *et al.* (1975) noted that human fibroblast interferon, induced with Newcastle disease virus, bound somewhat stronger to ω-aminohexyl agarose and *Lotus* lectin agarose than $(I)_n \cdot (C)_n$-induced interferon. This indicates a relatively higher hydrophobicity for virus than for $(I)_n \cdot (C)_n$-induced interferon. That both $(I)_n \cdot (C)_n$ and Newcastle disease virus-induced interferon may originate from the same genes (or set of genes) was suggested by Tan *et al.* (1974a) who showed in their study of mouse-human cell hybrids that human interferon production induced by $(I)_n \cdot (C)_n$ and by Newcastle disease virus segregated concordantly with chromosomes 2 and 5.

10. POLYNUCLEOTIDE FEATURES AND PROPERTIES THAT ACT AS DETERMINANTS FOR INTERFERON INDUCTION

10.1. STRANDEDNESS

Double-strandedness is a necessary but not sufficient condition for effective interferon induction by synthetic polynucleotides. The double-helix must be of the classical type involving two antiparallel helices with Watson–Crick base-pairing. This criterion does not imply that the double-helix must necessarily be formed from two separate strands as with $(I)_n \cdot (C)_n$, but rather that the backbone(s) of the nucleic acid is so arranged as to present a double-helical Watson–Crick base-paired structure. Thus, alternating copolymers such as $(A\text{–}U)_n$, $(I\text{–}C)_n$, $(A\text{–}br^5U)_n$ and $(1\text{–}br^5C)_n$, which can form hairpin double-helices by folding back on themselves, have been reported to be efficient interferon inducers (Colby and Chamberlin, 1969; De Clercq *et al.*, 1970b,e, 1971). There seems to be little restriction of base content or sequence for a dsRNA to act as an interferon inducer; thus, a variety of natural dsRNAs have been shown to be effective inducers (see Section 8). Table 8 presents the synthetic polynucleotides that have been reported to induce interferon.

Synthetic homopolynucleotides, random nucleotide copolymers and random nucleotide heteropolymers which form varying degrees of secondary structure, but are not regular double-helices, are either inactive or, at best, very poor interferon inducers. Several reports have appeared on the antiviral and interferon-inducing properties of the homopolymers $(I)_n$ and $(C)_n$ (Baron *et al.*, 1969; Billiau *et al.*, 1969). However, the activities of these polymers were considerably lower than that of

TABLE 8. *Synthetic Polynucleotides which have been Reported to Possess Significant Activity as Interferon Inducers*

Duplex	T_m (°C)[a]	Activity *in vitro*	Activity *in vivo*	Leading reference(s)	Comments
$(I)_n \cdot (C)_n$	63[b]	+	+	Tytell and Field, 1972 Rodgers and Merigan, 1972	To date, only synthetic polynucleotide inducers used in humans.
$(I)_n \cdot (m^5C)_n$	80[b]	+	ND	De Clercq *et al.*, 1972c	
$(I)_n \cdot (ff^5C)_n$	67[b]	+	ND	Folayan and Hutchinson, 1974	
$(I)_n \cdot (br^5C)_n$	89[d]	+	+	Torrence *et al.*, 1974 De Clercq *et al.*, 1976a	*In vitro* more active than $(I)_n \cdot (C)_n$ at low concentration (0.1–1.0 μg/ml).
$(I)_n \cdot (s^5C,C)_n$	64[e]	+	ND	O'Malley *et al.*, 1975	Activity falls with >1.7% 5-mercaptocytidylate
$(I)_n \cdot (s^5C)_n$	>100[b]	+	+	Reuss *et al.*, 1976	Complex is highly resistant to degradation by human serum.
$(A\text{–}s^2U)_n$	>100[b]	+	ND		
$(I)_n \cdot (C_x,U)_n$	var	+	+	Carter *et al.*, 1972, 1976	$x \geqslant 12$
$(I)_n \cdot (C_x,G)_n$	var	+	+	Ts'o *et al.*, 1976	$x \geqslant 20$
$(I_x,G)_n \cdot (C)_n$	var	ND	+	Matsuda *et al.*, 1971	Activity varies with x.
$(I)_n \cdot [(Cm)_x,C]_n$	var	+	ND	Merigan and Rottman, 1974	Activity depends on x.
$[(Im)_x,I]_n \cdot (C)_n$	var	+	ND		
$(G)_n \cdot (C)_n$	>100[f]	+	+	Colby and Chamberlin, 1969 Aksenov *et al.*, 1973 Timkovsky *et al.*, 1973 Novokhatsky *et al.*, 1975	Contrast with findings of Matsuda *et al.*, 1971; De Clercq *et al.*, 1970a and De Clercq and Torrence, 1977
$(c^7D)_n \cdot (C)_n$	49[d]	+	+	Torrence *et al.*, 1974	
$(c^7D)_n \cdot (br^5C)_n$	86[d]	+	+	De Clercq *et al.*, 1976a	
$(D)_n \cdot (C)_n$	50[e]	+	+	Black *et al.*, 1972	
$(sD)_n \cdot (C)_n$	62[e]	+	+	Black *et al.*, 1973	
$(sI)_n \cdot (sC)_n$	62[e]	+	+		Phosphorothioate modification increases resistance to nucleases
$(sI\text{–}sC)_n$	51[e]	+	+	De Clercq *et al.*, 1969, 1970b, c	
$(sA\text{–}sU)_n$	62[e]	+	+		
$(A\text{–}sU)_n$	68[f]	+	ND		
$(sI\text{–}C)_n$	50[e]	+	+		
$(A\text{–}br^5U)_n$	79[e]	+	ND	Colby and Chamberlin, 1969	
$(I\text{–}br^5C)_n$	86[e]	+	ND		
$(A\text{–}U)_n$	62[e]	+	−	De Clercq *et al.*, 1969, 1970c, 1971	*In vitro* activity may be enhanced by 'thermal activation'
$(I\text{–}C)_n$	47[e]	+	−		
$(G\text{–}C)_n$	92[e]	±	ND	De Clercq and Torrence, 1977	
$(I,m^2_2G)_n \cdot (C)_n$	var	+	ND	De Clercq *et al.*, 1975c	Depends on % of odd nucleotides
$(I,ms^2I)_n \cdot (C)_n$	var	+	ND		
$(ac^4C_3,C_7)_n \cdot (I)_n$	68	+	ND	Pitha and Pitha, 1971	

ND—not determined; var—variable T_m depending on per cent composition, see listed references.
[a] As given in cited reference or from the compilation by Janik (1971).
[b] $[Na^+] = 0.15$ M, pH 7.
[c] $[Na^+] = 0.10$ M, pH 7.
[d] $[Na^+] = 0.20$ M, pH 7.
[e] $[Na^+] = 0.15$ M, pH 7, $[Mg^{+2}] = 0.001$ M.
[f] $[Na^+] = 0.001$ M, pH 8.1, $[Mg^{+2}] = 0.5$ M.

$(I)_n \cdot (C)_n$; furthermore, the results depended heavily on the source of the homopolymer and the cell culture employed. Others have failed to confirm these results (e.g. Field *et al.*, 1968; De Clercq, 1974; Field, 1973). The minimal antiviral activity that can be manifested by 'single-stranded' homopolyribonucleotides appears to depend on the polymer's assumption of some degree of ordered structure or multistrandedness (Fig. 3). Thus, De Clercq and Merigan (1969) showed a correlation between antiviral activity of homopolynucleotides and those conditions that favor an 'ordered' structure. Consistent with its lack of ordered structure at room temperature or above (Felsenfeld and Miles, 1967; Michelson *et al.*, 1967), the polymer $(U)_n$ failed

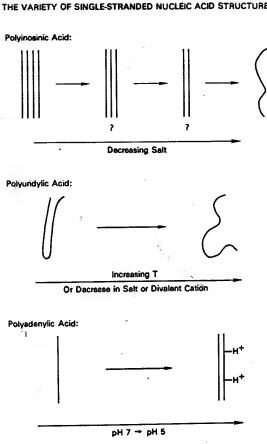


FIG. 3. Highly schematic presentation of the variety of different forms that 'single-stranded' homopolynucleotides may attain.

to provide antiviral resistance under any conditions, whereas 'ordered' structures (possibly three- or four-stranded structures) like $(G)_n$, $(I)_n$ and $(X)_n$ did provide significant protection at sufficiently high concentration (10^2–10^4 greater than required for $(I)_n \cdot (C)_n$). Additionally, it is held that $(A)_n$ and $(C)_n$ form twin-stranded double-helices at acid pH (Felsenfeld and Miles, 1967; Michelson *et al.*, 1967), and De Clercq and Merigan found that lowering the pH of the medium in which the polymers were exposed to the cells effected a significant increase in the polymers' antiviral activity. More recently, certain $(I)_n$ preparations have been found that induce *in vitro* and *in vivo* titers of interferon only slightly inferior to that achieved by $(I)_n \cdot (C)_n$ (Thang *et al.*, 1977). Although no C residues could be detected in these preparations, they did show a greater reactivity to dsRNA antisera than did inactive preparations of $(I)_n$. Importantly, the precise conditions required for production of such active $(I)_n$ polymers have not yet been defined.

Nucleic acid triple-helices, regardless of thermal stability or composition, all fail to induce interferon (Colby and Chamberlin 1969; De Clercq *et al.*, 1970, 1974b, 1975d; Torrence *et al.*, 1976; Torrence and De Clercq, 1977). In fact, the remarkable sensitivity of the interferon induction assay to the strandedness of a nucleic acid has been exploited to gain information about new triple-helices which are of interest for their potential role in chromosome structure (Crick, 1971; Pettijohn and Hecht, 1973) and regulation of genetic expression (Miller and Sobell, 1966; Britten and Davidson, 1969). This assay is based on the superinduction phenomenon as originally described independently in the laboratories of Vilček and Ho (Vilček *et al.*, 1969; Vilček, 1970a; Tan *et al.*, 1970, 1971b; Vilček and Ng, 1971) applied to primary rabbit kidney cells (De Clercq *et al.*, 1974c). This biological assay system, in conjunction with classical

physicochemical and biochemical techniques, established the existence of a variety of triple-helices (Table 9). Of particular interest are the $(U)_n \cdot (A)_n \cdot (I)_n$ and $(X)_n \cdot (A)_n \cdot (U)_n$ triplexes (Fig. 4), which represent the first such structures composed of three different heterocyclic bases (De Clercq *et al.*, 1975d; Torrence and De Clercq, 1977). The existence of such triplexes considerably widens the variety of possible triple-helical interactions that could be expected in nature.

TABLE 9. *Triple-helical Polynucleotides*[a] *that have been Established with the aid of Interferon-induction Assays*

$(U)_n \cdot (A)_n \cdot (I)_n$[b]
$(X)_n \cdot (A)_n \cdot (U)_n$[c]
$(rT)_n \cdot (A)_n \cdot (I)_n$[d]
$(rT)_n \cdot (A)_n \cdot (U)_n$[d]
$(br^5U)_n \cdot (A)_n \cdot (U)_n$[d]
$(br^5U)_n \cdot (A)_n \cdot (rT)_n$[d]
$(dUz)_n \cdot (A)_n \cdot (U)_n$[d]
$(rT)_n \cdot (A)_n \cdot (dUz)_n$[d]
$(dT)_n \cdot (A)_n \cdot (U)_n$[d]
$(U)_n \cdot (A)_n \cdot (dU)_n$[d]
$(Um)_n \cdot (A)_n \cdot (U)_n$[d]
$(dUf)_n \cdot (A)_n \cdot (U)_n$[e]
$(U)_n \cdot (A)_n \cdot (c^7I)_n$[f]
$(U)_n \cdot (A)_n \cdot (L)_n$[f]
$(rT)_n \cdot (A)_n \cdot (c^7I)_n$[f]
$(rT)_n \cdot (A)_n \cdot (L)_n$[f]
$(U)_n \cdot (A)_n \cdot (U_{.5}I)_n$[f] $\quad 0.1 \leq x \leq 10$

[a] The homopolymer written to the left of $(A)_n$ is involved in Watson–Crick hydrogen-bonding, whereas the homopolymer to the right of $(A)_n$ is involved in Hoogsteen hydrogen-bonding. These are hypothetical hydrogen-bonding formulations based on assumptions regarding melting profiles of these triplexes and the established hydrogen-bonding schemes for such triplexes as $(A)_n \cdot 2(I)_n$ and $(A)_n \cdot 2(U)_n$ as detailed by Arnott's group (Arnott and Bond, 1973a, b). Evidence for the proposed (De Clercq *et al.*, 1975d) formulation for $(U)_n \cdot (A)_n \cdot (I)_n$ triplex has been obtained immunochemically (Rainen and Stollar, 1977). All of the listed triplexes are inactive as interferon inducers; in addition, $(A)_n \cdot 2(U)_n$, $(A)_n \cdot 2(rT)_n$, $(A)_n \cdot 2(br^5U)_n$, $(A)_n \cdot 2(X)_n$, $(A)_n \cdot 2(I)_n$, and $(A)_n \cdot 2(dUz)_n$ also fail to induce interferon when tested in 'superinduced' primary rabbit kidney cells.

[b] De Clercq *et al.*, 1975d.

[c] Torrence and De Clercq, 1977.

[d] Torrence *et al.*, 1976a.

[e] De Clercq *et al.*, 1976b.

[f] Torrence and De Clercq, unpublished observations.

10.2. MELTING TEMPERATURE (T_m)

The melting temperature (also referred to as transition temperature or denaturation temperature) is defined as the temperature at which an observed physical parameter has changed midway between that characteristic for the helix and that characteristic for the coil. Usually at this temperature, one-half of the base-pairs of the helix have been disrupted. Since melting curves are not infinitely sharp but occur over a range of one to several degrees, there is a range in the vicinity of T_m where the helix is fractionally to fully disrupted. The apparent dependence of interferon induction on the T_m of nucleic acid helics can be used to argue that a double-stranded structure is involved in the initial step(s) leading to interferon production. If the double-stranded structure is a *conditio sine qua non* for induction, then it follows that the double-helix must be intact under the assay conditions employed. The data in support of this criterion of effective interferon inducers have been detailed previously by De Clercq (1974) and Pitha and Hutchinson (1977). Generally, there appear to be three ranges of helix T_m that have significantly different effects on interferon induction.

(a) Complexes with $T_m < 40°$ do not induce interferon or provide antiviral resistance beyond the level afforded by the constituent homopolymers.

(b) Complexes with T_m's greater than 40° but less than 60° can induce interferon but are considerably less effective than complexes of the third class (see below). Thus, the alternating copolymer $(I\text{–}C)_n$, with $T_m \sim 46°$ and the modified double-helix

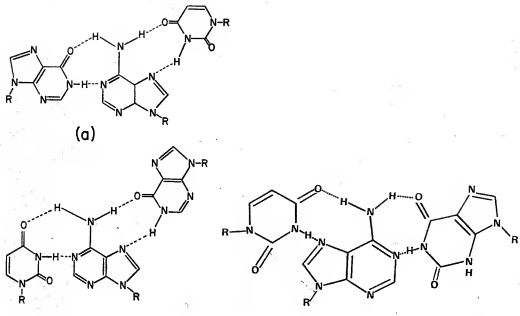


FIG. 4. Left: Two possible formulations of the polyadenylic·polyuridylic·polyinosinic acid triple-helix. Possibility b has been considered more likely on the basis of considerations discussed in the text. Right: A base-pairing formulation of the polyadenylic·polyxanthylic·polyuridylic acid triple-helix.

$(c^2I)_n \cdot (C)_n (T_m \sim 49°)$ definitely induce interferon but are 10–100 times less potent than $(I)_n \cdot (C)_n$ (De Clercq and Torrence, 1977; Torrence *et al.*, 1974). It is noteworthy that replacement of the 5-hydrogen of the cytosine bases by bromine in both cases elevates the T_m (by 20–30°) and simultaneously increases the interferon-inducing potency (Colby and Chamberlin, 1969; Torrence *et al.*, 1974).

(c) Experimentally, it is observed that the most active interferon inducers have T_m's $\sim 60°$ or greater. This is a *necessary* but *not sufficient* condition for maximal expression of antiviral activity and interferon inducing ability. Even within this class of inducers, there can be significant variations in the *in vitro* production of interferon when the polymers are examined in a dose-response approach. Thus at 0.1 μg/ml, $(I)_n \cdot (br^5C)_n$ led to the production of ten times more interferon in superinduced PRK cells than did $(I)_n \cdot (C)_n$. However, when evaluated at 10 μg/ml, both complexes gave comparable interferon titers (Torrence *et al.*, 1974; De Clercq *et al.*, 1976a).

Earlier accounts of the effect of nucleic acid T_m on interferon-inducing ability have considered only the T_m for the conversion of helix to coil (or double-stranded complex to constituent homopolymers or $T_m(2 \rightarrow 1)$). Later studies (De Clercq *et al.*, 1974b) suggest that interferon-induction is also dependent on $T_m(2 \rightarrow 3)$ or the temperature at which a double-helix rearranges to a triple-helix. This rearrangement is illustrated for the case of $(A)_n \cdot (U)_n$ (Stevens and Felsenfeld, 1964; Miles and Frazier, 1964; Blake *et al.*, 1967).

$$2(A)_n \cdot (U)_n \xrightarrow[T_m(2 \rightarrow 3)]{} (A)_n \cdot 2(U)_n + (A)_n$$

There exist conditions of ionic strength and divalent cation wherein, as the temperature is increased, the double-helical $(A)_n \cdot (U)_n$ will first rearrange to the triplex, and then at higher temperature the triplex will finally melt to the homopolymers. For all complexes like $(A)_n \cdot (U)_n$, which can form triple-helices, phase diagrams can be constructed (e.g. see Stevens and Felsenfeld, 1964) such that regions are found where one or the other helical form *alone* can exist *regardless* of the ratio of homopolymers. The connection of this physical property ($T_m(2 \rightarrow 3)$) to interferon induction was

inferred by a study (De Clercq *et al.*, 1974b) which showed that $(A)_n \cdot (br^5U)_n (T_m(2 \rightarrow 3) \sim 45°)$ did not induce interferon, $(A)_n \cdot (U)_n (T_m(2 \rightarrow 3) \sim 49°)$ was of intermediate activity, and $(A)_n \cdot (rT)_n (T_m(2 \rightarrow 3) \sim 53°)$ was even more active, comparable in some assays to $(I)_n \cdot (C)_n$. Thus, as with $(A)_n \cdot (br^5U)_n (T_m(2 \rightarrow 1) \sim 90°)$, a nucleic acid duplex may possess a high T_m, but fail to induce interferon due to rearrangement to triple-helix under physiological conditions. This strandwise $(2 \rightarrow 3)$ rearrangement is a factor to be considered only for duplexes that can form triple-helices. $(I)_n \cdot (C)_n$ and many of its analogs cannot do so.

One other recent illustration of the importance of $T_m(2 \rightarrow 1)$ in interferon induction is the case of polynucleotide displacement reactions. Sigler *et al.* (1962) provided evidence for the first known polynucleotide displacement reaction: namely,

$$(A)_n \cdot 2(I)_n + 2(C)_n \rightarrow (A)_n + 2(I)_n \cdot (C)_n$$
$$T_m = 44° \qquad T_m = 63°$$

They noted that the reaction proceeded in the direction of the helix with the higher T_m and that reaction occurred at temperatures well below the T_m of either helix. Subsequently, and in accordance with the above, it was found (De Clercq *et al.*, 1974c, 1976d) that when $(A)_n \cdot 2(I)_n$, itself an inactive inducer, was added to PRK cells together with $(C)_n$, an enormous (>2000 times) increase in interferon titer resulted. This technique, coupled with physicochemical studies, was used to demonstrate additional polynucleotide displacements (Table 10). In all instances, the reaction proceeded in the direction of the helix with the higher T_m and in the direction of the helix with the greater interferon-inducing activity.

10.3. Resistance to Degradation by Nucleolytic Enzymes

A number of studies have suggested that dsRNAs must have adequate resistance to degradation by nucleases; i.e. the helix must survive extra- and/or intracellular nucleases in order to reach the hypothetical receptor site. The preceding criterion regarding T_m may reflect this requirement. Duplexes with a low T_m, due to extensive 'breathing' of the double-helix, are readily attacked by single-strand endonucleases

TABLE 10. *Polynucleotide Displacement Reactions Suggested by Interferon Induction Assays and Confirmed by UV Absorbance-temperature Profiles*[a]

Reaction	T_m (°C) Reacting helix	T_m (°C) Resulting helix	
$(A)_n \cdot 2(I)_n + 2(C)_n \rightarrow (A)_n + 2(I)_n \cdot (C)_n$	44	63	(0.15 M Na^+)
$(A)_n \cdot 2(I)_n + 2(br^5C)_n \rightarrow (A)_n + 2(I)_n \cdot (br^5C)_n$	44	87	(0.15 M Na^+)
$(A)_n \cdot 2(c^7I)_n + 2(C)_n \leftrightarrow (A)_n + 2(c^7I)_n \cdot (C)_n$	47	47	(0.15 M Na^+)
$(A)_n \cdot 2(c^7I)_n + 2(br^5C) \rightarrow (A)_n + 2(c^7I) \cdot (br^5C)_n$	47	83	(0.15 M Na^+)
$(L)_n \cdot (C)_n + (I)_n \rightarrow (L)_n + (I)_n \cdot (C)_n$	47	67	(0.20 M Na^+)
$(L)_n \cdot (C)_n + (c^7I)_n \rightleftharpoons (L)_n + (c^7I)_n \cdot (C)_n$	47	49	(0.20 M Na^+)
$(L)_n \cdot (br^5C)_n + (I)_n \rightarrow (L)_n + (I)_n \cdot (br^5C)_n$	72	87	(0.15 M Na^+)
$(L)_n \cdot (br^5C)_n + (c^7I)_n \rightarrow (L)_n + (c^7I)_n \cdot (br^5C)_n$	72	83	(0.15 M Na^+)
$(I)_n \cdot (C)_n + (br^5C)_n \rightarrow (C)_n + (I)_n \cdot (br^5C)_n$	63	87	(0.15 M Na^+)
$(c^7I)_n \cdot (C)_n + (I)_n \rightarrow (c^7I)_n + (I)_n \cdot (C)_n$	49	67	(0.20 M Na^+)
$(c^7I)_n \cdot (C)_n + (br^5C)_n \rightarrow (C)_n + (c^7I)_n \cdot (br^5C)_n$	47	83	(0.15 M Na^+)
$(c^7A)_n \cdot (U)_n + (A)_n \rightarrow (c^7A)_n + (A)_n \cdot (U)_n$	32.5	56	(0.10 M Na^+)
$(c^7A)_n \cdot (U)_n + (rT)_n \rightarrow (c^7A)_n \cdot (rT)_n$	32.5	47	(0.10 M Na^+)
$(c^7A)_n \cdot (U)_n + (br^5U)_n \rightarrow (U)_n + (c^7A)_n \cdot (br^5U)_n$	32.5	69	(0.10 M Na^+)
$(c^7A)_n \cdot (rT)_n + (A)_n \rightarrow (c^7A)_n + (A)_n \cdot (rT)_n$	47	72	(0.10 M Na^+)
$(c^7A)_n \cdot (rT)_n + (br^5U)_n \rightarrow (rT)_n + (c^7A)_n \cdot (br^5U)_n$	47	69	(0.10 M Na^+)
$(c^7A)_n \cdot (I)_n + (C)_n \rightarrow (c^7A)_n + (I)_n \cdot (C)_n$	23	69	(0.45 M Na^+)
$(c^7A)_n \cdot (I)_n + (U)_n \rightarrow (I)_n + (c^7A)_n \cdot (U)_n$	23	43	(0.45 M Na^+)
$2(c^7A)_n \cdot (I)_n + (A)_n \rightarrow 2(c^7A)_n + (A)_n \cdot 2(I)_n$	23	51	(0.45 M Na^+)
$(c^7A)_n \cdot (br^5U)_n + (A)_n \rightarrow (c^7A)_n + (A)_n \cdot (br^5U)_n$	69	87	(0.10 M Na^+)

[a] De Clercq *et al.* (1976d).

For the first displacement reaction listed, see also Sigler *et al.* (1962). For the reaction of $(I)_n \cdot (C)_n$ with $(br^5C)_n$, see also Michelson *et al.* (1967).

wherever they present such denatured single-stranded sequences. The ubiquitous occurrence of intra- and/or extracellular endo- and exonucleases in a variety of animal cells, tissue and sera has been convincingly demonstrated (Shugar and Sierakowska, 1967; Bernard, 1969). Conceivably, double-stranded nucleic acids could be targets for attack by single-strand exonucleases (e.g. spleen phosphodiesterase), single-strand endonucleases (e.g. pancreatic ribonuclease A), aggregates of single-strand nucleases, e.g. aggregates of pancreatic ribonuclease (D'Alessio *et al.*, 1972; Libonati, 1971) or nucleases with a specificity toward dsRNA, like ribonuclease III of *E. coli* (Robertson *et al.*, 1968). Figure 5 presents some of the data relating interferon-inducing ability of a dsRNA to nuclease sensitivity in a format similar to that used earlier by De Clercq (1974). The alternating copolymers $(A\text{–}U)_n$ and $(I\text{–}C)_n$, before 'thermal activation', are rapidly degraded by pancreatic ribonuclease and are poor interferon inducers. When these polymers are subjected to 'thermal activation' or are chemically modified through the introduction of phosphorothioate residues, they achieve a much higher level of resistance to nuclease and also become more effective interferon inducers (reviewed by De Clercq, 1974). The duplex $(c^7I)_n{\cdot}(C)_n(T_m\ 49°)$ is more readily degraded by pancreatic RNase A than $(I)_n{\cdot}(C)_n(T_m\ 65°)$, and introduction of bromine into C–5 of cytidine increases the T_m (85°) and renders the complex more resistant to nuclease degradation, resulting in an inducer that is as effective as $(I)_n{\cdot}(C)_n$ (Torrence *et al.*, 1974; De Clercq *et al.*, 1976). On the other hand, increase of nuclease resistance above a minimal level does not have such dramatic effects: $(I)_n{\cdot}(br^5C)_n$ and $(I)_n{\cdot}(s^2C)_n$ are both much more nuclease resistant than $(I)_n{\cdot}(C)_n$, but do not provide commensurately higher titers of interferon or antiviral protection (Torrence *et al.*, 1974; De Clercq *et al.*, 1976a; Reuss *et al.*, 1976). Provision of the duplex RNAs with virtually absolute protection against degradation by pancreatic RNase by modification of the 2′-OH group has an effect just the opposite of that desired: all such complexes are totally inactive as interferon inducers (see below).

Consideration of these data alone leads to the conclusion that, like T_m or double-strandedness, nuclease resistance, *per se*, is not a sufficient requirement for interferon induction by synthetic polynucleotides. On the other hand, these data would suggest that some threshold level of nuclease resistance may be necessary for maximum expression of an inducer's potential.

It is possible that nuclease resistance may play an even more important role for *in vivo* induction of interferon, especially in humans and monkeys. When compared to experimental results from studies in mice or rabbits, both human and non-human primates seem to be relatively unresponsive to $(I)_n{\cdot}(C)_n$ (Hill *et al.*, 1971, 1972; Field *et al.*, 1971; De Vita *et al.*, 1970; Young, 1971). It is well established that human serum contains single-strand ribonuclease activity (e.g. Kamm and Smith, 1972; Sheid *et*

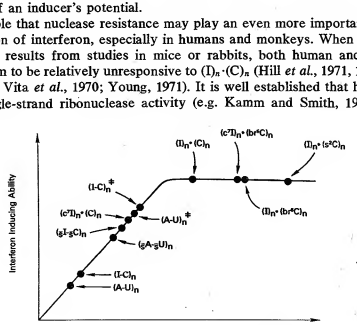


FIG. 5. Schematic representation of the dependence of interferon inducing ability on the inducer's resistance to degradation by nucleases. For $(I\text{–}C)_n$ and $(A\text{–}U)_n$, the superscript + denotes the effect of 'thermal activation' (Section 10.9.1) on their activity.

al., 1972; Lechner and Duque Magalhaes, 1973), and this activity may even be elevated in certain disease states (Reddi and Holland, 1976; Fink *et al.*, 1971; Chretien *et al.*, 1973; Houck and Berman, 1958). Furthermore, there is evidence that human serum and lymphocytes contain a ribonuclease activity that specifically degrades $(I)_n \cdot (C)_n$, and this activity is greater than that of single-strand ribonuclease (Nordlund *et al.*, 1970; Stern, 1970; Torelli *et al.*, 1976). Levy *et al.* (1975) have reported that a nuclease resistant complex of $(I)_n \cdot (C)_n$ with poly(L-lysine) and carboxymethylcellulose induced significant levels of interferon in rhesus monkeys and chimpanzees under conditions where $(I)_n \cdot (C)_n$ alone produced little or none. This particular $(I)_n \cdot (C)_n$ preparation, the exact nature of which has not been defined, proved useful in protecting rhesus monkeys against simian hemorrhagic fever, an invariably fatal disease to macaque monkeys (Levy *et al.*, 1976). This same inducer preparation also has been shown to suppress several markers (e.g. DNA polymerase, surface and core antigens) associated with chronic hepatitis B virus infection in chimpanzees (Purcell *et al.*, 1976).

10.4. The Effects of Strand Interruption and Molecular Weight on Interferon Induction by dsRNA

A variety of studies have addressed the relationship of the size of the $(I)_n \cdot (C)_n$ duplex or its constituent homopolymer strands to its ability to induce interferon. They fall into two classes:

(1) *Experiments in which the molecular size of the* $(I)_n \cdot (C)_n$ *complex as a whole was varied either by degradation (sonication or thermal) or by simultaneously reducing the molecular size of the individual homopolymer constituents.* These studies all agree that as the size of the duplex falls below some critical value, the antiviral activity and interferon-inducing ability of the duplex rapidly diminishes (Lampson *et al.*, 1970; Niblack and McCreary, 1971; Morahan *et al.*, 1972a; Shiokawa and Yaoi, 1972; Black *et al.*, 1973; Stewart and De Clercq, 1974; Machida *et al.*, 1976). Shiokawa and Yaoi (1972) claimed that very large molecular weight $(I)_n \cdot (C)_n$ possessed a somewhat reduced ability to induce interferon.

Similar results have been found using natural dsRNAs of varying molecular size (Gajdosova *et al.*, 1973; Kleinschmidt *et al.*, 1974; Edy *et al.*, 1974) (see Section 8).

(2) *Experiments in which the molecular size of one constituent homopolymer was held constant while the molecular size of the complementary partner was varied.* It is in these results that considerable conflict is apparent. On the one hand, Wacker *et al.* (1969) and Niblack and McCreary (1971) found that the decreased interferon inducing activity of various $(I)_n \cdot (C)_n$ complexes of the above type was independent of whether the $(I)_n$ component or $(C)_n$ component was the low molecular size constituent. On the other hand, numerous other investigations (Tytell *et al.*, 1970; Carter *et al.* 1972; Mohr *et al.*, 1972; Lampson *et al.*, 1972; Stewart and De Clercq, 1974) found that interferon-inducing ability was more dependent on maintaining a high molecular size of the $(I)_n$ strand than of the $(C)_n$ strand. The suggestion has been advanced (De Clercq, 1974) that these data are not really so conflicting since different investigators employed different molecular sizes of $(I)_n$ and $(C)_n$ in their studies. De Clercq (1974) points out that there is a critical range (2–5 *S*) in which there is a nearly linear decrease of interferon-inducing ability with decreasing molecular size of either component of $(I)_n \cdot (C)_n$. Wacker *et al.* (1969) studied complexes in which the molecular weight fell below the critical range of 2 *S* whereas Niblack and McCreary studied $(I)_n \cdot (C)_n$ complexes in which both strands fell into this critical range. Morahan *et al.* (1972a) used complexes of $(I)_n \cdot (C)_n$ which had molecular sizes above 5 *S*. The others (Tytell *et al.*, 1970; Carter *et al.*, 1972; Mohr *et al.*, 1972; Stewart and De Clercq, 1974) have maintained one strand of very high molecular size while varying the size of the second strand through values in this critical range.

These studies, in themselves, provide no rigorous evidence that interferon induction

is more sensitive to reduction in the molecular weight of the $(I)_n$ strand of $(I)_n \cdot (C)_n$ than to a similar reduction in the molecular weight of the $(C)_n$ strand. An equivalence of $s_{20,w}$ for samples of $(I)_n$ and $(C)_n$ does not imply equivalence of molecular weights. The sedimentation coefficient is given as

$$s = \frac{M(1 - \bar{v}_2\rho)}{N_A f}$$

where M is the molecular weight of the dry unsolvated macromolecule, N_A is avogadro's number, $\bar{v}_2$ is the thermodynamic partial specific volume of the solute, ρ is the solvent density, and f the frictional coefficient of the macromolecule (Tanford, 1961). The deduction of M from s requires consideration of both $\bar{v}_2$ and f which are molecular parameters related to the form the macromolecule assumes under the conditions employed for sedimentation. Both $(I)_n$ and $(C)_n$ take on dramatically different macromolecular forms under most conditions. For instance, at pH 7, 0.15 M NaCl and 20°, $(C)_n$ is likely a single-stranded base-stacked structure (Bloomfield *et al.*, 1974), whereas $(I)_n$ is probably a four-stranded helix (Souleil and Panijel, 1968; Thiele and Guschlbauer, 1973; Zimmerman *et al.*, 1975; Arnott *et al.*, 1974a). Therefore, the use of any single empirically derived equation of the form $s_{20,w} = KM^a$ (where K and a are derived from experiment) to relate the sedimentation coefficient of $(I)_n$ or $(C)_n$ to molecular weight is unjustified. Even application of the Flory–Mandelkern equation that relates $s_{20,w}$, M and intrinsic viscosity is not possible since unwarranted assumptions regarding the macromolecular form must be made. The equation has the form

$$M = \left[\frac{s_{20,w}[\eta]^{1/3}\eta_0 N_A 10^{-13}}{\beta(1 - \bar{v}\rho)}\right]$$

where, in addition to the parameters defined above, η_0 is solvent viscosity, $[\eta]$ is intrinsic viscosity of the macromolecule, and β is a coefficient relating M to sedimentation viscosity. The value taken for β is critical since it was determined for flexible polymers as 2.5×10^6. When Eigner and Doty (1965) did their classical work on the relation of $s_{20,w}$ and $[\eta]$ to the M for native DNA, they first determined that β was reasonably constant and equal to the stated value for a variety of DNAs the M of which was determined by independent methods (e.g. light scattering).

Two separate reports have used a somewhat different approach to this question regarding the influence of strand interruption on interferon induction by dsRNAs (Carter *et al.*, 1972; Pitha and Carter, 1971a). This work relied upon oligoinosinate·poly(C) complexes and oligocytidylate·poly(I) complexes. The oligoinosinates were prepared by limited alkaline hydrolysis of poly(I), and the oligomers were chemically and enzymatically characterized to determine chain length (Tazawa *et al.*, 1972). The oligocytidylates were obtained by digestion of $(C_{20},G)_n$ by T_1 RNase. The products $(Cp)_n$ G > p were characterized by determination of the cytidine/guanosine ratio. The methodology employed to determine chain length is therefore independent of the conformation of the oligomer. The results of these investigations can be summarized as follows:

(a) Interruption of one of the two strands of the duplex reduced the interferon-inducing activity. $(I)_n \cdot (Cp)_{48}G > p$ was ten-fold less active than $(I)_n \cdot (C_{20},G)_n$, and $(C)_n \cdot (Ip)_{18}I$ was nearly inactive as an inducer even though its T_m is ~50°.

(b) The $(I)_n \cdot$oligoC duplex was a better inducer than the $(C)_n \cdot$oligoI duplex even for complexes with similar oligomer chain length and duplex T_m. For instance, $(I)_n \cdot (Cp)_{23}G > p$ was ten times more active as an inducer than was $(C)_n \cdot (Ip)_{18}I$. Thus, a reduction in the molecular weight (or chain length) of the $(I)_n$ strand of $(I)_n \cdot (C)_n$ leads to a more dramatic effect on the interferon-inducing activity of the complex than a similar reduction in the chain length of the $(C)_n$ strand of $(I)_n \cdot (C)_n$.

10.5. Dependence of Interferon Induction on the Nature of the Ribose–Phosphate Backbone of the Nucleic Acid (Fig. 6)

Phosphorothioate Analogs

Substitution of phosphorothioate  for phosphate can significantly increase the interferon-inducing ability of the alternating copolymers, $(A–U)_n$ and $(I–C)_n$ (De Clerq *et al.*, 1969, 1970b,c). When a similar modification is carried out on the $(I)_n$ and/or $(C)_n$ strand of $(I)_n \cdot (C)_n$, however, no such increase in activity occurs (Black *et al.*, 1972, 1973).

Modification of the 2′-Hydroxyl Group

To date, all attempts to totally replace the 2′-hydroxyl group of one or both strands of $(I)_n \cdot (C)_n$ or $(A)_n \cdot (U)_n$ with any substituent invariably produced duplexes with little, if any, interferon inducing ability. Modifications thus far executed include: 2′-fluoro (De Clercq and Janik, 1973), 2′-azido (Torrence *et al.*, 1973), 2′-chloro (Black *et al.*, 1972, 1973), 2′-*O*-methyl (De Clercq *et al.*, 1972c) and 2′-*O*-ethyl (De Clercq *et al.*, 1974a) in the $(U)_n$ strand of $(A)_n \cdot (U)_n$; 2′-hydrogen(deoxyribopolymers) (Vilček *et al.*, 1968; Colby and Chamberlin, 1969; De Clercq *et al.*, 1972c; Black *et al.*, 1972), 2′-chloro (Black *et al.*, 1972), 2′-azido (De Clercq and Hobbs, unpublished observations) and 2′-*O*-methyl (De Clercq *et al.*, 1972c; Merigan and Rottman, 1974) in the $(C)_n$ strand of $(I)_n \cdot (C)_n$; 2′-*O*-ethyl (De Clercq *et al.*, 1974a) in the $(A)_n$ strand of $(A)_n \cdot (U)_n$; 2′-methyl (Merigan and Rottman, 1974), 2′-hydrogen (Vilček *et al.*, 1968; Colby and Chamberlin, 1969; Hutchinson *et al.*, 1974) in the $(I)_n$ strand of either $(I)_n \cdot (C)_n$ or pyrimidine 5-substituted $(I)_n \cdot (C)_n$ analogs. Two separate studies have examined the effect of partial replacement of the 2′-hydroxyl group on interferon induction. Steward *et al.* (1972) prepared various 2′-*O*-acetyl derivatives of $(A)_n$, $(I)_n$ and $(C)_n$ by reaction of the corresponding homopolynucleotide with acetic anhydride. This treatment, according to the conditions employed, effected varying degrees of 2′-*O*-acetylation. When the acetylated polymers were mixed with the corresponding potentially complementary polynucleotide, those polymers with a high degree (>50%) of 2′-*O*-acetylation failed to form stable complexes ($T_m < 20°$) and to induce interferon. An $(A)_n \cdot (U)_n$ derivative in which 30 per cent of the 2′-OH groups of $(A)_n$ were acetylated provided marginal resistance to viral infection, and an $(I)_n \cdot (C)_n$ derivative with 6 per cent of the $(I)_n$ residues acetylated was just as active as $(I)_n \cdot (C)_n$ itself. Merigan and Rottman (1974) synthesized various copolymers of IMP and 2′-*O*-methyl IMP and CMP and 2′-*O*-methyl CMP with the aid of polynucleotide phosphorylase.

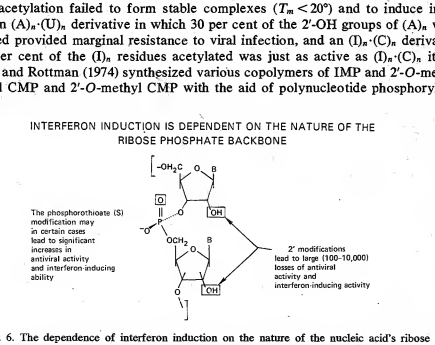


Fig. 6. The dependence of interferon induction on the nature of the nucleic acid's ribose phosphate backbone.

These partially methylated copolymers formed stable complexes with either $(I)_n$ or $(C)_n$. The partially methylated copolymer complexes could still provide antiviral resistance and induce interferon even when a substantial percentage of the 2'-OH groups were methylated. There was a definite decrease in antiviral activity with increasing methylation, but the complex $[(I_m)_4.\ I_1]_n\cdot(C)_n$ was nearly as active as $(I)_n\cdot(C)_n$and significantly more active than $[(Cm)_9,C]_n\cdot(I)_n$ or $[(Cm)_7,C_3]_n\cdot(I)_n$ in inducing interferon. However, the $[(Im)_4,I_1]_n\cdot(C)_n$ complex studied by Merigan and Rottman (1974) had a distinctly higher molecular size (6.5 *S*) than the $[(Cm)_9,\ C_1]_n\cdot(I)_n$ and $[(Cm)_7,C_3]_n\cdot(I)_n$ complexes (3.4 and 3.6 *S* respectively). These molecular size differences, situated in the critical zone, render interpretations as to the relative importance of 2'-*O*-methyl substitutions in the $(I)_n$ or $(C)_n$ strand of $(I)_n\cdot(C)_n$ rather difficult. A report which has not yet appeared in print has extended these observations and additionally suggests that 'clustering' of the Im residues in the (Im,I) copolymers decreased the interferon titers less than when the 2'-*O*-methylated residues were randomly inserted in the $(I)_n$ chain (Ts'o *et al.*, 1975). These results are not at all at variance with the aforementioned studies of 2'-modified polynucleotides. That a partially 2'-*O*-methylated copolymer complex can be an effective inducer of interferon might be expected from the distribution of sequences generated in such a random copolymer. The probability of finding a sequence of pure (Im) residues in the $[(Im)_4,I]_n$ copolymer is, for instance, only ~0.10 for a run of 11 nucleotide units (one turn of an A–RNA double-helix) so that these polymers do not at all closely resemble the 2'-modified homopolymers in which 100 per cent of the residues of one or both duplex strands are altered. In fact, the majority (~90 per cent) of sequences in these partially methylated polymers like $[(Im)_4,I]_n$ contain one or more unaltered 2'-hydroxyl groups.

10.6. Dependence of Interferon Induction on the Nature of the Heterocyclic Bases in the Interior of the Double-helix

The nature of the bases in the interior of the double-helix determines the ability of the resulting double-helix to induce interferon (Figs 7 and 8). Duplexes based on poly(7-deazaadenylic acid), $(c^7A)_n$, even though fulfilling all other established requirements for effective inducers (such as high T_m, appropriate molecular size, double-strandedness, resistance to nucleases and the presence of 2'-hydroxyl groups), fail to induce interferon or provide antiviral protection (De Clercq *et al.*, 1974b; Torrence and Witkop, 1975). In contrast, introduction of the same modification

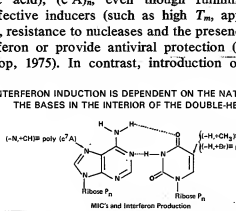
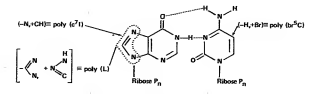


FIG. 7. The dependence of the interferon inducing ability of a dsRNA on the nature of the heterocyclic bases in the interior of the helix: effect of modifications to $(A)_n\cdot(U)_n$. In this figure, the alternative acceptable abbreviation for nucleic acids used, i.e. poly(A) = $(A)_n$. Abbreviations used are MIC (minimum inhibitory concentration; that concentration which effects a 50 per cent reduction in virus cytopathogenicity), PRK (primary rabbit cells) and VSV (vesicular stomatitis virus). Interferon production is measured in international units/ml.

INTERFERON INDUCTION IS DEPENDENT ON THE NATURE OF THE BASES IN THE INTERIOR OF THE DOUBLE-HELIX



MIC's and Interferon Production

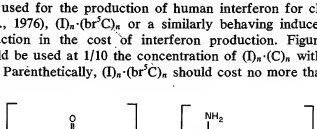
Polymer	MIC (μg/ml)*	Interferon Production†
poly (I) · poly (C)	0.003 – 0.001	3000
poly (I) · poly (br^{5}C)	0.003 – 0.0003	3000
poly (c^{7}I) · poly (C)	0.3	600
poly (c^{7}I) · poly (br^{5}C)	0.003 – 0.0003	3000
poly (L) · poly (C)	>10	<3
poly (L) · poly (br^{5}C)	>10	<3

*In PFK cells with VSV challenge.
†Polymers applied at 10μg/ml.

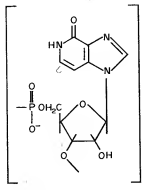
FIG. 8. The dependence of the interferon inducing ability of a dsRNA on the nature of the heterocyclic bases in the interior of the helix: effect of modifications to $(I)_n \cdot (C)_n$. See Fig. 7 for explanations and abbreviations.

(purine N7→CH) into the purine ring of inosine to give poly(7-deazainosinic acid), $(c^7I)_n$, leads to complexes with excellent *in vitro* or *in vivo* interferon inducing activity (Torrence *et al.*, 1974; De Clercq *et al.*, 1976a). Double-stranded complexes based on the C-nucleoside, laurusin (formycin B), are inactive as inducers, even though they too fulfill the requirements for active inducers (Torrence *et al.*, 1975a). Finally, the polyxanthylic acid derived purine–purine duplex, $(A)_n \cdot (X)_n$ ($T_m \sim 85°$), did not induce interferon or antiviral resistance (Torrence *et al.*, 1976b), the double-helical complexes of poly(3-isoadenylic acid) (Michelson *et al.*, 1966) with either poly(I) or poly(U) (T_m's $\sim 80°$ in 0.01 M salt) failed to induce interferon in mice (Uchic, 1975), and a complex of poly(3-deazainosinic acid) with $(br^5C)_n$ of rather low T_m (52° in 0.15 M salt) did not induce interferon (De Clercq *et al.*, 1976c).

An additional point of interest arose in the study of the interferon inducing ability of the $(c^7I)_n$ derived helices. Both $(c^7I)_n \cdot (br^5C)_n$ and $(I)_n \cdot (br^5C)_n$ brought about the production of higher titers of interferon at low concentrations than did $(I)_n \cdot (C)_n$ in 'superinduced' primary rabbit kidney cells and human skin fibroblasts (Fig. 9) (Torrence *et al.*, 1974; De Clercq *et al.*, 1976a). Since this same fibroblast 'superinduction' system is used for the production of human interferon for clinical trials (e.g. Desmyter *et al.*, 1976), $(I)_n \cdot (br^5C)_n$ or a similarly behaving inducer could effect a significant reduction in the cost of interferon production. Figure 9 shows that $(I)_n \cdot (br^5C)_n$ could be used at 1/10 the concentration of $(I)_n \cdot (C)_n$ with no decrease of interferon titer. Parenthetically, $(I)_n \cdot (br^5C)_n$ should cost no more than $(I)_n \cdot (C)_n$ since



Scheme 1.



Poly (3-deazainosinic acid)

Scheme 2.

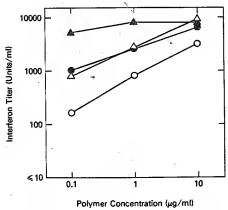


FIG. 9. Comparison of the ability of $(c^7I)_n \cdot (C)_n$, (○); $(c^7I)_n \cdot (br^5C)_n$, (●); $(I)_n \cdot (C)_n$, (△); $(I)_n \cdot (br^5C)_n$, (▲) to induce interferon in human skin fibroblasts 'superinduced' with cycloheximidine and actinomycin D (De Clercq *et al.*, 1976a).

the only difference in its synthesis is the chemical bromination of CDP (e.g. Howard *et al.*, 1969). It might also be instructive to compare the activity of $(I)_n \cdot (br^5C)_n$ with $(I)_n \cdot (C)_n$ in an *in vivo* situation where nuclease destruction of the inducer is considered of importance (Levy *et al.*, 1975). $(I)_n \cdot (br^5C)_n$ is more resistant to pancreatic ribonuclease A than $(I)_n \cdot (C)_n$; furthermore, its apparent ability to produce high titers of interferon at lower concentrations than $(I)_n \cdot (C)_n$ might be advantageous.

While dealing with base-modified polynucleotides, the interferon-inducing capacity of $(G)_n \cdot (C)_n$ should be discussed. There is considerable disagreement in the literature regarding the inducing ability of this complex. Field *et al.* (1967b). Matsuda *et al.* (1971), Field (1973), De Clercq *et al.* (1970a) and Gresser (1969), (unpublished observations, cited in De Clercq *et al.*, 1970a) found that mixtures of $(G)_n$ and $(C)_n$ did not induce interferon *in vivo* or *in vitro* although as admitted by these authors, there was no hypochromicity observed on mixing the individual homopolymers. Colby and Chamberlin (1969) reported that $(G)_n \cdot (C)_n$, synthesized from a $(C)_n$ template with RNA polymerase, could induce interferon in DEAE-dextran treated chick embryo cells, and more recently, several publications have suggested that $(G)_n \cdot (C)_n$ formed from the separate homopolymers, can induce interferon *in vivo* and *in vitro* if careful attention has been devoted to purity, molecular weight and annealling conditions for the complex (Novokhatsky *et al.*, 1975; Aksenov *et al.*, 1972; Timkowsky *et al.*, 1973; Subbotina *et al.*, 1972). Nonetheless, a number of attempts using the above recommended purification and annealling procedures as well as the annealling procedures used by Pochon and Michelson (1965) and Englander *et al.* (1972), failed to give a complex that could induce interferon *in vitro* even though the Englander procedure

gave rise to a clearly defined 1:1 $(G)_n \cdot (C)_n$ complex (Torrence and De Clercq, unpublished observations). The difficulties surrounding $(G)_n \cdot (C)_n$ are closely related to the extremely stable secondary structure of $(G)_n$ (e.g. Fresco and Massoulié, 1963; Guschlbauer, 1972). It is possible that synthesis of $(G)_n \cdot (C)_n$ on a $(C)_n$ template (Haselkorn and Fox, 1965) may circumvent this problem, and this is why Colby and Chamberlin (1969) found $(G)_n \cdot (C)_n$ to induce interferon.*

10.7. Effects of Base-mismatching on the Antiviral and Interferon-inducing Properties of Synthetic Polynucleotides

'Base-mismatching' is defined as the opposition in a nucleic acid double-helix of two bases which cannot form normal Watson–Crick base-pairs; thus, the opposition of the complementary bases adenine and uracil in a double-helix leads to the classical Watson–Crick base-pair, but the opposition of, for instance, the noncomplementary bases uracil and cytosine or adenine and cytosine results in 'mismatching'. The ability of 'mismatched' duplexes to induce interferon may be discussed in terms of the probable structure of such polymers. First, it can be reasonably assumed that all the copolymers dealt with herein are *random* in nature. Copolymers synthesized in this same way (with polynucleotide phosphorylase) were used to elucidate the genetic code dictionary (Watson, 1976). However, some copolymers (e.g. $(A,U)_n$) may not be completely random; instead, the alternating A–U sequence may predominate (Simka and Zimmerman, 1960). Second, on the basis of experimental evidence and model building, it has been established that opposing non-complementary pyrimidine residues cannot take up the intrahelical conformation in a classical Watson–Crick double-helix. Rather they consistently form a helix-with-loops structure *regardless of base composition* (Fig. 10) (Lomant and Fresco, 1975; Topal and Fresco, 1976). Third, again on the basis of experiment and model building, opposing non-complementary pyrimidine–purine pairs and purine–purine pairs can assume *intrahelical* conformations depending on both the quantity of non-complementary base present in the helix and the helical environment (temperature, ionic strength, divalent metal cation, etc.) (Fig. 10) (Lomant and Fresco, 1975; Topal and Fresco, 1976; Wang and Kallenbach, 1971).

When we attempt to relate these findings to studies on interferon induction, two complications arise.

(1) Due to the aforementioned environmental effect, cases will arise in which there will be ambiguity as to whether the 'mismatched base' is extrahelical or intrahelical under conditions of interferon induction. This will occur as the mole fraction of the odd base reaches some critical value. An intrahelical base may become extrahelical if the frequency of the 'mismatched' base becomes sufficient to disrupt the stacking interactions that maintain the bases in register (Wang and Kallenbach, 1971).

(2) The nature of these copolymer derived complexes does not necessarily lead to an unambiguous interpretation; i.e. the finding, for instance, that a complex containing mismatched but intrahelical bases induces interferon does not necessarily imply that such mispairs do not affect ability to induce interferon. These are random copolymers; therefore, regardless of composition, there will be a real probability of existence of an uninterrupted run of undisturbed Watson–Crick base-pairs.

Two separate inquiries into the effect of base-mismatching on interferon induction have been conducted. The first (Matsuda *et al.*, 1972) employed a large number of copolymer-derived complexes, some of which were studied more intensively by Carter *et al.* (1972). Several major difficulties are, however, associated with the earlier study: no melting profile data were presented to give an indication of the persistence of the complexes under physiological conditions; no sedimentation values were reported for the polymers to permit differentiation of effects due to molecular weight as opposed to composition; none of the involved copolymers were analyzed to

* Note that Colby and Chamberlin actually measured resistance to sindbis virus infection, not interferon production *per se*.

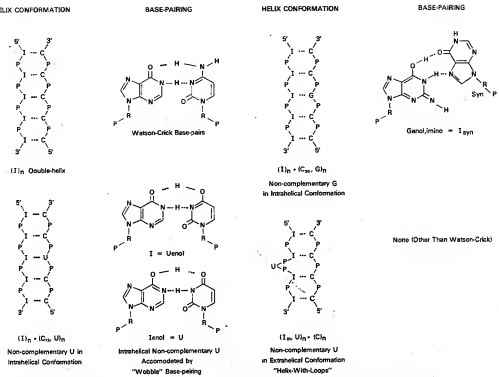


FIG. 10. 'Unwound' double-helices showing the various possible conformations of noncomplementary bases and the mode of base-pairing by which they may be accommodated under the restraints of the classical Watson–Crick double-helix. These representations are based on the data and conclusions of Lomant and Fresco (1975) and Topal and Fresco (1976). The notation Uenol, for instance, indicated that uridine has assumed the rare enol tautomeric form. I_{syn} indicates that the base-sugar torsion angle of inosine is in the syn range, opposite of the anti conformation that predominates at equilibrium.

determine the critical ratios of nucleosides. The indicated copolymer composition was deduced directly from the input diphosphate ratio in the polynucleotide phosphorylase reaction mixture, an unjustified assumption that can lead to significant errors in composition, especially with all copolymers containing guanosine (Grunberg-Manago, 1963). A final problem lies in the reconciliation of Matsuda *et al.*'s data with the data of other investigators; no *in vitro* data were reported in this study, only *in vivo* data. All other studies of structure-activity relationships have been done with cell culture *in vitro*. The most obvious and most concrete conclusion that can be drawn from these data (Matsuda *et al.*, 1971) is that ability to induce interferon decreases with increasing content of the odd mismatched base. The result obtained with these mismatched analogs stands in contrast to the results obtained for the series $(I_xG)_n \cdot (C)_n$ where both inosine and guanosine residues are engaged in Watson–Crick base-pairs with cytidine. For these polymers, the interferon titer increased with increasing G content to a level of 33 mole per cent G (Matsuda *et al.*, 1971).

The experimental finding of Carter *et al.*, (1972) showed that the incorporation of a non-complementary base in the $(I)_n$ strand of $(I)_n \cdot (C)_n$ virtually abolished the antiviral activity and interferon-inducing activity of $(I)_n \cdot (C)_n$, but the corresponding properties of $(I)_n \cdot (C_x,U)_n$ polymers depended on the ratio of C to U in the $(C)_n$ strand. Additionally, one complex containing mismatched G residues, namely $(I)_n \cdot (C_{20},G)_n$, was also nearly as active as $(I)_n \cdot (C)_n$. This decrease in antiviral activity of some of the complexes could not be ascribed to their increased nuclease sensitivity (see below) since complex formation with polylysine, although considerably increasing nuclease resistance to degradation by pancreatic ribonuclease, did not enhance the activity of any of the inactive duplexes. Furthermore, the melting temperatures of the duplexes were sufficiently high to insure that the helices would persist under physiological conditions.

The most dramatic result of Table 11 is that $(I_{21},U)_n \cdot (C)_n$ and $(I_{39},U)_n \cdot (C)_n$ provided only marginal antiviral protection and induced no interferon. The U residues in these complexes are extrahelically situated and lead to a helix-with-loops structure (Lomant and Fresco, 1975; Topal and Fresco, 1976). The data on the $(C,U)_n \cdot (I)_n$ complexes is more difficult to interpret. On the one hand, the interferon inducing ability of the polymers decreases with increasing fraction of mismatched base and with decreasing probability of finding an intact $(I)_n \cdot (C)_n$ sequence for recognition by a receptor. There may also, however, be evidence in this series that an extrahelical nucleotide residue is counterproductive to interferon induction. Although the $(I)_n \cdot (C_x,U)_n$ copolymers in Table 11 have been shown to exist as helices in which the mismatched bases are accommodated intrahelically (Wang and Kallenbach, 1971), it is possible that in the case of the poor inducer, $(I)_n \cdot (C_4,U)_n$, the U residues may rearrange to the extrahelical conformation since this complex has a comparatively low T_m. On the whole, however, the bulk of these data suggest that an intrahelical mismatch does not have as dramatic an effect on interferon induction as does an extrahelical mismatch.

If, as will be discussed later, interferon induction depends on the recognition of a particular conformational arrangement of the polynucleotide, then the differential effect of extrahelical versus intrahelical mismatches can be rationalized. Since the necessary information for recognition by the hypothetical receptor could possibly lie within one or less than one helical turn, but since the $(I_x,U)_n \cdot (C)_n$ duplexes contain a sufficient number of such determinants, the inactivity of these polymers may not be ascribed directly to a failure of the extrahelical residue to be accommodated at the receptor's binding site. The looped-out structure does not distort the remainder of the helix. Rather, it is possible that the loop may interfere (e.g. through steric or electrostatic forces) with the proper alignment or approach of the intact unaltered helical segments with the receptor. Mismatched, but intrahelical residues do not affect induction so much since these mismatches can be accommodated in the Watson–Crick helix with little or no helical distortion at the site of their introduction (Topal and Fresco, 1976); i.e. they do not negatively affect the approach of the remainder of the helix to the receptor. It is even possible that regions containing intrahelical purine–pyrimidine mispairs might interact with the receptor site just as effectively as the regions containing only classical Watson–Crick pairs.

Independent of theoretical considerations for the behavior of these mismatched analogs, the above data, coupled with consideration of the extra- or intrahelical state of mismatched bases, cannot be interpreted as implying any greater importance of the $(I)_n$ strand *vis à vis* the $(C)_n$ strand in the induction process. Rather, the differential behavior of the above mismatched polymers may be viewed as a consequence of the

TABLE 11. *The Effect of Base Mismatching on the Antiviral Activity and Interferon-inducing Ability of Synthetic Nucleic Acids*

Double-helical RNA	T_m[a]	MID[b]	Interferon production[c]	Probability of undecameric sequence
$(I_{21},U)_n \cdot (C)_n$	59	$\sim 10^{-3}$ M	0	0.602
$(I_{39},U)_n \cdot (C)_n$	62	$\sim 10^{-4}$ M	0	0.725
$(I)_n \cdot (C_1,U_1)_n$		$>10^{-3}$ M	0	0.0005
$(I)_n \cdot (C_4,U)_n$	49	$>10^{-3}$ M	0	0.095
$(I)_n \cdot (C_7,U)_n$	57	$\sim 10^{-4}$ M	5	0.235
$(I)_n \cdot (C_{13},U)_n$	60	$<10^{-5}$ M	10–20	0.434
$(I)_n \cdot (C_{22},U)_n$	62	$<10^{-5}$ M	10–20	0.602
$(I)_n \cdot (C_{25},G)_n$	61	$<10^{-5}$ M	5	0.60
$(I)_n \cdot (C)_n$	65	$<10^{-5}$ M	20–40	

[a] As determined in minimal Eagle's salt solution.

[b] Concentration required to inhibit viral cytopathogenicity by 50 per cent. Estimated from Carter *et al.*, 1972.

[c] Polymers were applied to the cells for 1 hr at 37°C. The cells were then decanted free and washed 3 times and further incubated for 18 hr before the supernatant fluids were withdrawn for titration. Results expressed in terms of international reference units/ml.

ability of the $(C)_n$ strand to intrahelically accommodate either odd pyrimidine or purine bases. In contrast, the $(I)_n$ strand can intrahelically accommodate only odd purine introductions. The base-pairing schemes defined by Topal and Fresco (1976) thus lead to fundamental physical differences in the helices, and these differences are perceived by the receptor site for polynucleotide interferon inducers.

Carter *et al.* rejected the extrahelical versus intrahelical configuration as a cause for the differential behavior of these analogs on the basis of the activity of one complex; namely, $(I)_n \cdot (C_{20},G)_n$. They suggested that this complex forms a helix-with-loops structure in analogy to the series $(I)_n \cdot (C_{4\text{-}12},I)_n$ (Wang and Kallenbach, 1971). Consideration of recent evidence suggests that the G residues in $(I)_n \cdot (C_{20},G)_n$ may assume the intrahelical state: first, the results of Wang and Kallenbach were obtained under conditions that do not favor intrahelical conformations (low salt, no divalent cation); second, the studied polymers contained a much greater quantity of odd base than does $(I)_n \cdot (C_{20},G)_n$: third, guanine, due to its greater stacking ability, has a greater tendency to form intrahelical structures than does hypoxanthine (Lomant and Fresco, 1975); finally, both G·G oppositions and G·A oppositions have been experimentally demonstrated in model helices, and it is possible to write similar base-pairing schemes for the G·I opposition (Fig. 10). Clearly, additional experimental inquires will be necessary to provide a final resolution to this question.

Carter and his colleagues also investigated the hydrolysis of these mismatched $(I)_n \cdot (C)_n$ analogs by ribonucleases; specifically, a mixture of RNase I_1 and pancreatic RNase A (Carter *et al.*, 1972). Both the $(I)_n \cdot (C_{13},U)_n$ and the $(I)_n \cdot (C_{20},G)_n$ duplexes were degraded at a faster rate than was $(I)_n \cdot (C)_n$. This result is to be expected in view of the nature of mismatched bases. Normally, both T_1 RNase and pancreatic ribonuclease degrade only single-stranded nucleic acids after G_p (or I_p) or pyrimidine residues, respectively. At the higher concentrations employed in the present experiments, both enzymes can degrade double-stranded nucleic acids. There is excellent evidence that they do so by simply denaturing a region of the double-helix or binding to the single-stranded region when the helix 'breathes' and then acting on the single-stranded region as they would on a normally single-stranded polymer. Pancreatic RNase can lower the T_m of a double-helical polymer (Von Hippel and McGhee, 1972); furthermore, the degradation of double-helical homopolymers by pancreatic RNase A correlates well with their T_m. The higher melting the duplex, the slower the degradation (Torrence and Witkop, 1975; Torrence *et al.*, 1975a). Although the mismatched residue in $(I)_n \cdot (C_{20},G)_n$ and $(I)_n \cdot (C_{13},U)_n$ are intrahelically disposed, the mispaired residues are destabilized relative to the Watson–Crick pairs; e.g. I·U pair is destabilized by 1.7 kcal/mole from an I·C pair (Wang and Kallenbach, 1971). Such regions in the double-helix therefore breathe more readily, and RNase can bind to and attack these residues more frequently than it could in the all Watson–Crick helix.

It has been abundantly demonstrated that the triggering of interferon production by polynucleotides is a remarkably rapid process (De Clercq *et al.*, 1971; Billiau *et al.*, 1972b; Pitha *et al.*, 1972). Having demonstrated that certain mismatched duplexes could induce interferon nearly as well as $(I)_n \cdot (C)_n$ but at the same time were more rapidly degraded by nucleases, Carter *et al.* (1972) suggested that these mismatched analogs might show a more favorable therapeutic index *if* the 'toxic' manifestations of dsRNA required a longer period for development. Thus was introduced the concept of temporal modification of interferon inducer toxicity.

In recent studies (Ts'o *et al.*, 1976; Carter *et al.*, 1976) this hypothesis was explored in detail by examination of the relative toxicities of these mismatched analogs compared to $(I)_n \cdot (C)_n$. The results can be summarized as follows:

(a) The complexes $(I)_n \cdot (C_{13},U)_n$, $(I)_n \cdot (C_{12},U)_n$, $(I)_n \cdot (C_{29},G)_n$ and $(I)_n \cdot (C_{20},G)_n$ were as effective as $(I)_n \cdot (C)_n$ in protecting mice against the lethal effects of Semliki Forest virus infection when the polymers were given at 100 or 25 μg/animal. The guanosine containing duplexes were somewhat less effective at 10 μg/animal. $(I)_n \cdot (C_{29},G)_n$ was also less effective in protection against encephalomyocarditis virus infection.

(b) The acute toxicity (in mice) of the analogs decreased in the order $(I)_n \cdot (C)_n$ $(I)_n \cdot (C_{13},U)_n$ $(I)_n \cdot (C_{20},G)_n$.

(c) As measured with spleen cells, the mismatched polymers $(I)_n \cdot (C_{12},U)_n$ and $(I)_n \cdot (C_{29},G)_n$ appeared to be somewhat less mitogenic than was $(I)_n \cdot (C)_n$.

(d) Under conditions where all the mismatched complexes induced high levels of circulating interferon in rabbits, they were dramatically less pyrogenic than $(I)_n \cdot (C)_n$.

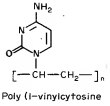
(e) Chronic toxicity studies showed that the analogs $(I)_n \cdot (C_{29},G)_n$ and $(I)_n \cdot (C_{12},U)_n$ were less toxic than $(I)_n \cdot (C)_n$ in reducing body weight or inducing thymic atrophy; furthermore, in contrast to the other polymers, $(I)_n \cdot (C_{12},U)_n$ did not induce anemia or changes in neutrophil count. In other aspects (spleen weight, lymphocytopenia, thrombocytopenia), the mismatched analogs and $(I)_n \cdot (C)_n$ behaved similarly.

(f) Finally, evidence was presented that $(I)_n \cdot (C_{29},G)_n$ appeared to induce antibody at a slower rate than did $(I)_n \cdot (C)_n$, although after several weeks, the level of antibody was the same as with $(I)_n \cdot (C)_n$. In contrast, $(I)_n(C_{12},U)_n$ appeared to induce only very low levels of antibody throughout the time period examined. Experimentally, this observation was obtained by immunization of both mice and rabbits with the various duplexes and then assaying antigen binding capacity using radio-labeled $(I)_n \cdot (C)_n$ as the antigen. The authors claimed to show cross-reactivity by experiments with passive cutaneous anaphylaxis in guinea pigs. However, antisera to dsRNAs like $(A)_n \cdot (U)_n$ cross react (measured by complement fixation) well with natural dsRNAs or $(I)_n \cdot (C)_n$ but show significant, often dramatic, differences in immuno-reactivity upon minor structural alterations of dsRNA (Stollar, 1973; Stollar, 1975; Johnston *et al.*, 1975; Lacour *et al.*, 1973). Furthermore, passive cutaneous anaphylaxis will not provide a quantitative measure of cross-reactivity, since both serum and antigen are employed in massive amounts.

In toto, these two studies (Ts'o *et al.*, 1976; Carter *et al.*, 1976) can be taken to indicate that base-mismatching in dsRNAs can lead to a diminution of at least several (acute and chronic toxicity, pyrogenicity) toxic reactions of dsRNA interferon inducers. These conclusions are important considerations for the design of therapeutically useful polynucleotides as inducers of interferon since they imply that manipulation of the biological half-life of inducers may decrease the toxic manifestations without significantly compromising the interferon-inducing and thus the antiviral properties of the nucleic acids.

10.8. Induction of Interferon by Hypermodified Polynucleotide Analogs

In view of the exquisite sensitivity of interferon induction to small changes in nucleic acid structure, the report (Pitha and Pitha, 1971) that a nonhelical complex of polyinosinic acid with poly(1-vinylcytosine) could provide antiviral resistance and induce interferon appears to contradict the established stringent structural requirements. The deviation of the structure of this complex from double-helical polynucleotides is enormous: the vinylcytosine polymer contains no sugar backbone and is



Poly (1-vinylcytosine

Scheme 3.

electrically neutral; it readily forms large aggregates and is not a 1:1 complex like $(I)_n \cdot (C)_n$, but carries a 4:1 ratio of cytosine to hypoxanthine bases. Its activity is even more surprising in view of the observation that an $(I)_n \cdot (C)_n$ analog containing a

random distribution of 2′→5′ phosphodiester linkages in the $(C)_n$ strand is totally inactive (Pitha and Pitha, 1971). The suggestion has, however, been advanced (Pitha and Pitha, 1974) that the poly(1-vinylcytosine) partner of the complex acts to enhance the marginal antiviral activity (Baron *et al.*, 1969; De Clercq and Merigan, 1969) of the normal $(I)_n$ strand in much the same manner as polybasic substances like DEAE-dextran (Billiau *et al.*, 1969). Pitha and Pitha (1974) compared the interferon-inducing ability of complexes of various cytosine substituted poly(D-lysines) and poly(L-lysines) with $(I)_n$ or $(C)_n$ to complexes of $(I)_n$ and $(C)_n$ with unsubstituted parent polycations. These complexes formed at 1:1 stoichiometric ratios of polycation to polyanion regardless of the cytosine content of the modified poly(lysine), and complexes were formed with polynucleotides bearing either complementary or non-complementary bases, suggesting that charge neutralization, rather than hydrogen bonding, was the determinant of complex formation. The cytosine substituted poly(D-lysine) polymers enhanced the antiviral activity of *both* $(I)_n$ and $(C)_n$; furthermore, the poly(L-lysine) polycations (substituted with cytosine or unsubstituted) showed little, if any, enhancing effect. These results indicated that the biological activity of polynucleotide–polycation complexes described above, is dependent chiefly on the nature of the polycation itself and the formation of aggregates, rather than the presence of complementary base-pairing.

10.9. ADDITIONAL CONSIDERATIONS

10.9.1. *'Thermal Activation'*

When preincubated at 37° in minimal Eagle's medium (MEM), several nucleic acids, including the alternating-copolymers $(A\text{–}U)_n$, $(I\text{–}C)_n$, $(G\text{–}C)_n$ and homopolynucleotide duplexes, $(A)_n\cdot(U)_n$ and $(I)_n\cdot(C)_n$, became markedly more active (10^2–10^5 times) in reducing vesicular stomatitis plaque formation in human skin fibroblasts and some other cell cultures. In contrast, preincubation at 0° in MEM had no such effect. (De Clercq *et al.*, 1970e, 1971; De Clercq and Merigan, 1971). This process required divalent metal cation and was concentration dependent (at higher concentrations, the polynucleotide underwent the activation less effectively). Both 'activated' and 'unactivated' preparations of $(A\text{–}U)_n$ were equally sensitive to exonuclease degradation, but the 'activated' $(A\text{–}U)_n$ was markedly more resistant to endonuclease degradation. The 'activated' $(A\text{–}U)_n$ also appeared to bind to cells at a greater rate than did the 'unactivated' polymer; in addition, the former persisted at the cell surface longer than did the latter. Physical studies, including u.v. spectroscopy, viscosity, analytical sedimentation velocity and cesium sulfate buoyant density, failed to reveal any significant differences between the 'activated' and 'unactivated' polynucleotides. Several hypotheses have been advanced to explain these observations (De Clercq *et al.*, 1971) including direct or indirect slippage that might rearrange base-pairing to eliminate unpaired regions. Nonetheless, no direct proof for any structural mechanism for this phenomenon has so far been presented.

10.9.2. *Sequential Administration of Polynucleotides*

De Clercq and De Somer (1971a, 1972a) found that if $(I)_n$ was added to cells, the cells washed, and $(C)_n$ was added, or if the reverse protocol was followed, an antiviral activity resulted which equalled or surpassed the activity of $(I)_n\cdot(C)_n$ itself administered as the intact complex. In a sense, $(I)_n$ may be said to prime the cells for the antiviral activity of $(C)_n$ and vice versa. The following experiments were offered to support the hypothesis that the homopolymers reunited at the cellular level to form the active $(I)_n\cdot(C)_n$ duplex and ruled out the possibility that the homopolymers might be functioning independently.

(a) When applied in succession onto the cells, only polymers that were complementary were effective in providing antiviral resistance; e.g. $(I)_n$ followed by $(G)_n$ produced no effect.

(b) The priming effect of $(I)_n$ was destroyed when the cells were treated with T_1 ribonuclease prior to $(C)_n$ treatment, and the priming effect of $(C)_n$ was destroyed when the cells were treated with pancreatic ribonuclease prior to treatment with $(I)_n$.

(c) While pancreatic ribonuclease could render acid soluble the radioactivity of $[^3H]$–$(C)_n$ when it was bound to untreated cells, it did not acid-solubilize the label of $[^3H]$–$(C)_n$ when it was applied to cells pretreated with $(I)_n$. Only when very high concentrations of ribonuclease (40 μg/ml) were applied was $(C)_n$ degraded.

(d) $[^3H]$–$(C)_n$ became cell associated more quickly when the cells were previously exposed to $(I)_n$ than when they were left untreated.

These data support the conclusion that upon sequential administration, the complementary polymers reunite at the cellular level, possibly at the cell membrane. Noteworthy was the observation that the antiviral activity was retained even when the cells, after exposure to one homopolymer $[(I)_n]$, were incubated in polymer free medium for 24 hr before addition of the second polymer $(C)_n$.

This effect of sequential administration of homopolymers was not always observed; thus, the sequences $(A)_n$ then $(U)_n$ or $(U)_n$ then $(A)_n$, and the sequences $(I)_n$ then $(br^5C)_n$, or $(c^7I)_n$ then $(br^5C)_n$, did not give antiviral protection equal to or greater than the parent duplexes (De Clercq *et al.*, 1974b; Torrence *et al.*, 1974b).

The observation that the antiviral activity from the sequential administration of $(I)_n$ and $(C)_n$ exceeded the activity of the intact $(I)_n \cdot (C)_n$ duplex held only under the special condition that $(I)_n$ was added in excess of the $(C)_n$ strand and that $(I)_n$ was applied before $(C)_n$ (De Clercq and De Somer, 1971a, 1972a).

In a later study, De Clercq *et al.* (1973a) established that the addition of $(I)_n$ to the cells, followed by $(C)_n$, gave an interferon response that was more resistant to inhibition by poly(L-lysine) or pancreatic ribonuclease treatment than when the polymers were applied in the reverse order. In addition, poly(L-lysine) removed more label from cells treated with $[^3H]$–$(C)_n$ followed by $(I)_n$ than from cells treated with polymers in the reverse order. These data were interpreted as implying that the sequence $(I)_n$ followed by $(C)_n$ gave rise to $(I)_n \cdot (C)_n$ that was more firmly cell-associated than when $(C)_n$ was applied before $(I)_n$.

Sequential administration of single-stranded and double-stranded polymers has also been investigated (De Clercq *et al.*, 1974c, 1975d, 1976d; Torrence *et al.*, 1976a). In these experiments, either a decrease or an increase in interferon production was noted, depending on whether a polynucleotide displacement reaction occurred or a triple-helix was formed. When various homopolymers (e.g. $(U)_n$, $(rT)_n$, $(dUz)_n$, $(I)_n$) were added to the cells as a mixture with duplex polynucleotides (e.g. $(A)_n \cdot (U)_n$, $(A)_n \cdot (rT)_n$), a dramatic decrease of the interferon inducing capacity of the active duplexes was observed (Table 12). The basis for this phenomenon was determined to be the formation of triple-helical polymers (e.g. $(U)_n \cdot (A)_n \cdot (I)_n$, $(U)_n \cdot (A)_n \cdot (U)_n$) which are unable to induce interferon. When the homopolymer was added to the cells (in serum-free medium) for 1 hr before addition of $(A)_n \cdot (U)_n$, such polymers as $(dUz)_n$, $(dT)_n$, $(Um)_n$ or $(I)_n$ could still effect a large reduction in interferon titer, while ribohomopolymers $((U)_n$, $(rT)_n$, $(br^5U)_n)$ did not lead to a substantial reduction in interferon titer. When the homopolymer was added to the cells after they had been exposed for 1 hr to the active duplex $(A)_n \cdot (U)_n$, all polynucleotides could still bring about a reduction in interferon titer, but the reduction was less pronounced compared to when the homopolymers and the active duplex were added simultaneously. The latter finding, that homopolymers could reduce interferon production even when added 1 hr after the active duplexes, is not in disagreement with earlier work that showed 1 hr or less exposure of cells to inducer is sufficient for maximal antiviral protection and for interferon mRNA translation (De Clercq *et al.*, 1971; Pitha *et al.*, 1972; Reynolds and Pitha, 1974; Vengris *et al.*, 1975).

(a) For experiments in which addition of the homopolymers 1 hr after the active duplex effectively reduced the interferon response to the active duplex, $(A)_n \cdot (U)_n$ was generally used as the active inducer. In studies concerning the minimal exposure time required for full interferon production, $(I)_n \cdot (C)_n$ has generally been used as the

TABLE 12. *The Effect of Simultaneous and Sequential Administration of Homopolynucleotides and their Complexes on Interferon Production in Primary Rabbit Kidney Cells Superinduced with Cycloheximide and Actinomycin D*[a]

System	Method of addition	Interferon titer Fraction of Control
$(I)_n + (A)_n \cdot (U)_n$	$(I)_n + (A)_n \cdot (U)_n$	0.02
	$(I)_n$ then $(A)_n \cdot (U)_n$	0.03
	$(A)_n \cdot (U)_n$ then $(I)_n$	0.30
$(U)_n + (A)_n \cdot (U)_n$	$(U)_n + (A)_n \cdot (U)_n$	0.05
	$(U)_n$ then $(A)_n \cdot (U)_n$	1.0
	$(A)_n \cdot (U)_n$ then $(U)_n$	0.1
$(dUz)_n + (A)_n \cdot (U)_n$	$(dUz)_n + (A)_n \cdot (U)_n$	0.01
	$(dUz)_n$ then $(A)_n \cdot (U)_n$	0.007
	$(A)_n \cdot (U)_n$ then $(dUz)_n$	0.20

[a] Data taken from Torrence *et al.*, 1976a; De Clercq *et al.*, 1975d. Homopolymer and homopolymer duplex were either: (a) mixed to give a final concentration of 5 and 10 μg/ml, respectively, and then incubated for 1 hr at 37°C in MEM before addition to the cells; or (b) added sequentially (each at 10 μg/ml in MEM) to the cells in a 1 hr interval. A control value was determined by adding MEM in place of the homopolymer in either the sequential administration or the experiments in which the polymers were added as a mixture. See De Clercq *et al.* (1974b) for experimental details of the subsequent superinduction procedure.

inducer. The time requirements for both complexes to induce interferon are not necessarily identical.

(b) Addition of the homopolymers after the inducer resulted in a less effective reduction in interferon than when polymers were applied together.

(c) Under these conditions, both poly(L-lysine) and pancreatic ribonuclease reduced interferon production or antiviral activity of $(I)_n \cdot (C)_n$ when they were added as late as one hour after exposure of cells to the inducer (De Clercq *et al.*, 1973a).

The finding that homopolymers added first to the cell could bring about a large reduction in interferon titer deserves further comment. Other studies have shown that, depending on cell type and concentration, 0.5–10 per cent of applied double-helical or single-stranded polynucleotides bind to the cells and is not removed by washing (Pitha and Carter, 1971; De Clercq and De Somer, 1972a, 1973b; Bausek and Merigan, 1969). Similar binding of the homopolymers probably occurs in the sequential administration experiments; thus, when the homopolymer is added to the cells first, >90 per cent of it is removed by washing. When the active duplex is added to the cells, it is therefore in considerable excess, and even though triplex-helix formation occurs with the homopolymer bound to the cell, a large percentage of the inducing duplex would remain unaffected and would be expected to induce interferon. The fact that little, if any, interferon is produced under these conditions suggests that (a) the triple-helical complex formed inhibits the triggering of the interferon response to the active duplex; or (b) the single-stranded polymer itself inhibits triggering by the active duplex, and triple-helix formation is not involved. The latter possibility seems unlikely since these same homopolymers do not affect interferon induction by $(I)_n \cdot (C)_n$ or $(A)_n \cdot (U)_n$ when no triplex formation is possible (De Clercq *et al.*, 1974b). The former explanation seems more accurate, especially since triplexes themselves, when applied first in sequential administration experiments, can inhibit interferon production by active duplexes (e.g. $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$ (De Clercq *et al.*, 1974b and see below)).

When applied to the cells prior to active inducers, homopolynucleotides possess different efficiencies in reducing interferon production. Ribohomopolymers, like $(U)_n$, $(rT)_n$, and $(br^5U)_n$, are comparatively inefficient, but 2'-modified polymers like $(dUz)_n$, $(dT)_n$, $(dU)_n$, $(dUfl)_n$ and $(Um)_n$, and the purine ribopolymers, $(X)_n$ and $(I)_n$, are quite effective. This suggests that the former polymers are not available to react with the active double-helix whereas the latter are able to do so. It is noteworthy that the latter polymers are all highly resistant to nucleases (e.g. pancreatic RNase A) whereas the former are not. Hence, ribohomopolymers, such as $(U)_n$, $(rT)_n$, and $(br^5U)_n$, may be

degraded by cell-associated nucleases during the 1 hr interval that precedes the addition of the active duplex $(A)_n \cdot (U)_n$.

10.9.3. *Potentiation of Interferon Induction by Polycationic and Basic Substances*

A number of basic substances of high and low molecular weight have been shown to increase interferon production *in vitro* (Table 13). *In vivo* potentiation of interferon production has been claimed for DEAE-dextran (Dianzani *et al.*, 1969), poly(D-lysine) (Rice *et al.*, 1970), and poly(L-lysine) (Levy *et al.*, 1975). The potentiation of interferon production by polylysine in mice is difficult to relate to the similar effect seen in monkeys (Levy *et al.*, 1975), since the latter supposedly have a high level of nuclease that destroys $(I)_n \cdot (C)_n$ whereas the former do not (Nordlund *et al.*, 1970). Whether or not potentiation is seen *in vitro* seems to depend on the assay conditions. DEAE-dextran has been reported to stimulate interferon induction by $(I)_n \cdot (C)_n$ in chick embryo cells (Colby and Chamberlin, 1969) only when serum is present in the incubation medium, not when serum is omitted (Machida *et al.*, 1975). Also, poly(L-lysine) has been reported to decrease the antiviral activity of $(A)_n \cdot (U)_n$ (De Clercq and Janik, 1973), and neomycin can do the same to $(I)_n \cdot (C)_n$ (Lampson *et al.*, 1969). The mechanism of the enhancing effect of DEAE-dextran on interferon production remains obscure. At least three origins of this phenomenon must be considered.

(a) Numerous studies have established that polyamines can bind to nucleic acids, more strongly to helix than to coil forms (Bloomfield *et al.*, 1974). One consequence of this effect is an increased resistance to degradation by nuclease (Lampson *et al.*, 1969;

TABLE 13. *Enhancement of Interferon Production by Polybasic Materials*

Enhancer	*In vitro*	*In vivo*	References
diethylaminoethyldextran	+	+	Dianzani *et al.*, 1968 Colby and Chamberlin, 1969 Billiau *et al.*, 1969 Dianzani *et al.*, 1969, 1971 Tilles, 1970 Pitha and Carter, 1971b Vilček *et al.*, 1972 Pitha and Pitha, 1974 Bausek and Merigan, 1969 De Clercq and Merigan, 1971a Falcoff and Perez-Bercoff, 1969 Mécs and Rosztóczy, 1971 Richmond, 1971b Kalmakoff and Austin, 1973 De Clercq and De Somer, 1975 Machida *et al.*, 1975 Long and Burke, 1971
neomycin	+	−	Billiau *et al.*, 1969 Lampson *et al.*, 1969
neamine	+	ND	Lampson *et al.*, 1969
streptomycin	+	ND	Billiau *et al.*, 1969 Lampson *et al.*, 1969
methylated albumin	+	ND	Billiau *et al.*, 1969
protamine sulfate	+	ND	Billiau *et al.*, 1969
poly(L-lysine)	+[a]	+	Kótai *et al.*, 1975 Levy *et al.*, 1975 Pitha and Pitha, 1974
poly(D-lysine)	+	+	Rice *et al.*, 1970, 1971 Pitha and Pitha, 1974
poly(L-ornithine)	+	ND	Wacker *et al.*, 1972
poly(dimethylaminoethyl glutamine)	+	+	Gánti *et al.*, 1976 Kótai *et al.*, 1975
poly(diethylaminoethyl glutamine)	+	ND	Kótai *et al.*, 1975
amphotericine	+	ND	Borden and Leonhardt, 1976
ionenes	+, −	ND	De Clercq *et al.*, 1977

[a] De Clercq and Janik (1973) found poly(L-lysine) decreased the antiviral activity of $(A)_n \cdot (U)_n$.
ND—not determined.

Dianzani *et al.*, 1971; Pitha and Carter, 1971b), but this can vary considerably depending on the ratio of polycation to polynucleotide (Pitha and Carter, 1971b).

(b) Whether or not enhancement of interferon production is witnessed depends on the ratio of the DEAE-dextran to nucleic acid (Pitha and Carter, 1971b; Tilles, 1970). Pitha and Carter found a maximal effect when an electroneutral complex between the polyanion and polycation was formed. DEAE-dextran also increased the uptake of $(I)_n \cdot (C)_n$ by cells (Pitha and Carter 1971b; Bausek and Merigan, 1969; Colby and Chamberlin, 1969) suggesting that increased uptake of polynucleotide might be involved in the enhancement process. It has not been determined with certainty, however, whether this increased uptake represents penetration of the polynucleotide across the cell membrane or simply a cell surface adsorption. Generally, however, there appears to be little relation between cell binding of nucleic acids and interferon induction (Bausek and Merigan, 1969).

(c) Another possibility is that polybasic substances might alter the cell membrane to expose occult membrane structures involved in the initial steps of the induction process. These sites need not bring about changes in gross binding characteristics since only a small number of them might be involved. That polybasic substances like DEAE-dextran would interact with the cell membrane seems probable in view of the fact that cell electrophoresis studies have demonstrated the presence of a considerable density of negatively charged groups on the surfaces of many cells (Mehrishi, 1972). Even $(I)_n \cdot (C)_n$, through presumed binding to positively charged membrane components, changes the electrophoretic mobility of cells (Mehrishi, 1970). If DEAE-dextran binds to cells, it would neutralize negatively charged areas of the membrane, thereby facilitating the electrostatic binding of $(I)_n \cdot (C)_n$ to the cell directly, or indirectly through provision of the excess positive charges provided by its own binding.

10.9.4. *Inhibition of Interferon Production by Polynucleotides*

Not only do polynucleotides induce interferon, they also inhibit its induction. Three classes of polynucleotide inhibitors of interferon induction can presently be described.

Class 1. Prevention of interferon induction due to a specific interaction of the polynucleotide with the inducing molecule: e.g. $(I)_n$ by formation of $(U)_n \cdot (A)_n \cdot (I)_n$ triplex (De Clercq *et al.*, 1975d) or $(U)_n$ and its various analogs by formation of triple-helices of the $(A)_n \cdot 2(U)_n$ class (Torrence *et al.*, 1976a).

Class 2. In contrast to the first class of inhibitors which are specific for one type of inducer [i.e. $(I)_n$ inhibits induction by $(A)_n \cdot (U)_n$ but not by $(I)_n \cdot (C)_n$], the second class of polynucleotides nonspecifically inhibits interferon induction by both $(A)_n \cdot (U)_n$ or $(I)_n \cdot (C)_n$ by virtue of their antimetabolic activities (De Clercq *et al.*, 1975a, 1976a, d). Thus, $(c^7A)_n$ inhibits interferon production by inhibition of host cell RNA synthesis, since the polymer is degraded to the nucleoside antibiotic, tubercidin (or its mononucleotide) which is a potent inhibitor of cellular macromolecular synthesis (Suhadolnik, 1970).

Class 3. Evidence has been accumulated for yet a third class of nucleic acid inhibitors of interferon induction. The mechanism(s) of action of this class of inhibitors has not yet been defined. The relevant studies are summarized in Table 14. It would appear that some disagreement exists regarding whether or not single-stranded polynucleotides can block induction. To establish a blocking effect, Kawade and Ujihara (1969) used a large excess (10 times) of the inactive RNA, and the resulting inhibition (assessed by increase of viral plaque number) was relatively small. Johnston *et al.* (1976) monitored the interaction of polynucleotides with the cell at 4°; it is well established that membranes can undergo phase transitions as the temperature changes (e.g. Fox and Keith, 1972). For this third class of polynucleotide inhibitors, De Clercq *et al.* (1974b, 1975e) offered a unifying hypothesis that the inactivity of single-stranded versus triple-helical and inactive double-helical polymers might have

TABLE 14. *Antagonizing Effect of Nucleic Acids on Interferon Induction by Synthetic Polynucleotides*

Reference	Cell system	Observed antagonizing effect
Kawade and Ujihara, 1969	Chick embryo fibroblasts	Non-inducing single-stranded RNAs inhibited plaque reduction by the active inducers $(I)_n \cdot (C)_n$ and MS2 phage RNA.
De Clercq *et al.*, 1974b, 1975e	Sequential administration in superinduced PRK cells	Single-stranded RNAs did not reduce interferon titer of $(A)_n \cdot (U)_n$ or $(I)_n \cdot (C)_n$ unless they were of class 1 inhibitors. Inactive duplexes and triplexes did effect reduction in interferon titer.
O'Malley *et al.*, 1975	Sequential administration in human skin fibroblasts	Single-stranded RNAs did not reduce the interferon titer of active inducers–two consecutive exposures to active inducers reduced the resultant interferon production.
Johnston *et al.*, 1976	Human skin fibroblast	Cells, exposed to $(I)_n \cdot (C)_n$ at 4°, lost their ability to produce interferon when the cells were washed with high concentrations of salt or incubated with single-stranded polynucleotides.
Torrence and De Clercq, 1977	Simultaneous administration in superinduced PRK cells	$(X)_n$ and its complexes reduced interferon induction by $(I)_n \cdot (C)_n$.

different causes. Single-stranded polynucleotides are inactive inducers because they fail to bind to the receptor site for interferon inducers. Inactive duplexes and triplexes, however, may bind to the postulated receptor, but fail to trigger the message for interferon production because they cannot undergo or cannot induce a required conformational attitude. In a further extension of these observations, it was shown that 2′-modified polymers fail to affect the titer of active inducers (De Clercq *et al.*, 1974a, 1975e). Thus, these duplexes also fail to induce interferon because they, like single-stranded polymers, do not bind to the hypothetical receptor. Those polynucleotides which do not bind to the postulated receptor site (e.g. single-stranded polynucleotides and 2′-substituted double-stranded complexes) may be assumed not to counteract the interferon-inducing capacity of the active double-stranded complexes, at least not when assayed under physiological conditions. However, triple-helical complexes which bind to the receptor site (albeit inadequately to trigger an interferon response) may effectively counteract (by such mechanisms as steric hindrance or electrostatic repulsion) the triggering process of active interferon inducers.

10.10. Considerations Concerning the Dependence of Interferon Induction on Nucleic Acid Structure

The sensitivity of interferon induction to structural features of synthetic polynucleotides can reveal a great deal about how nucleic acid inducers *do not* function and provide important insights into how they *may* function.

First, the possibility that polynucleotide inducers function through a kind of non-specific alteration of the cell can confidently be eliminated. Such dramatic dependence on structure, as is witnessed for nucleic acid interferon inducers, would not be expected for a substance which acts merely through accumulation by cells due to some favorable physical property as observed with depressants (hypnotics, general anesthetics). This possibility is also rendered improbable by the consideration, discussed earlier, that there is no correlation between cell uptake (or binding) of polynucleotide and ability to induce interferon. What remains to be established, however, is whether or not other activities associated with polynucleotides (e.g. pyrogenicity, immunoadjuvant effect, etc.) may be due to relatively non-specific alterations of membranes of reticulo-endothelial cells.

That both strands of the double-helical polynucleotide are not necessary for induction, but rather that one strand simply acts as a carrier for the other which is the true inducer, also seems improbable in view of the following considerations:

(a) A variety of different naturally occurring dsRNAs (as viral genomes or replicative intermediates) of varying base content and composition can induce interferon (see Section 8). Although not unequivocally established, dsRNA may be the signal for interferon production during the course of a viral infection (Carter and De Clercq, 1974). There would not seem to be any *a priori* reason to expect (or hypothetical mechanism to account for) discrimination of one strand of a double-helical nucleic acid from the other unless the recognition involved a highly specific base sequence, and this latter possibility is ruled out by data enumerated below.

(b) If the purpose of one of the two strands is merely to facilitate delivery (through protection against nuclease or increased uptake) of the complementary strand to the site where the triggering of interferon production occurs, then we might expect some cellular mechanism for the denaturation of the complex in the cell. This might be accomplished by something like a 'melting protein' which could recognize and bind to some single-stranded base sequence, an unlikely possibility for reasons discussed below. If, however, denaturation were involved, then an additional dependence on nucleic acid T_m should develop. Higher melting duplexes should be less active than lower melting duplexes since the former are less readily denatured. In other words, once the T_m of the complex has reached a value that permits adequate protection and facilitates uptake of the other strand (say ~60°), additional stability would be deleterious. To the contrary, dsRNAs with a T_m significantly higher than 60° [e.g. $(I)_n \cdot (br^5C)_n$] are at least as active in inducing interferon as $(I)_n \cdot (C)_n$ ($T_m \sim 60°$).

(c) If delivery of a single-strand of the duplex were all that is required, the form for carrying the relevant strand may be independent of helical state. For instance, for cells in which $(A)_n \cdot (U)_n$ can induce interferon, $(A)_n \cdot 2(U)_n$ ought to be just as effective, if $(A)_n$ were the inducer. Additionally, $(A)_n \cdot 2(I)_n$ and $(U)_n \cdot (A)_n \cdot (I)_n$ might be expected to be active inducers if $(C)_n$ simply functions as a carrier for $(I)_n$ in $(I)_n \cdot (C)_n$. These triple helices have relatively low T_m's for the dissociation of the $(I)_n$ strands (40°–60°, depending on salt concentration (De Clercq *et al.*, 1975).

(d) If delivery of a single-strand of the duplex were all that is required, then the interferon-inducing activity of the complex should be independent of the chemical nature of the carrier strand. The observation that $(I)_n$ complexed to 2'-modified $(C)_n$ polymers gives inactive duplexes is not consistent with such an expectation. It would be possible to circumvent this objection by invoking a type of ribonuclease assisted transport into the cell (Pitha and Hutchinson, 1977), but such a hypothesis would not seem consistent with the effects of DEAE-dextran, polylysine or other polycations on the activity of $(I)_n$.

(e) If there existed some mechanism to discriminate between the two strands of the dsRNA double-helix, then the recognition mechanism could not be based on the sugar–phosphate backbone, but only on the bases themsleves. The concepts involved in recognition of nucleotide residues have been dealt with extensively (Jovin, 1976; Von Hippel and Mc Ghee, 1972; Yarus, 1969), and the sites which could be employed by a protein for recognition of a base are illustrated in Fig. 11. Recognition of one strand over the other could not be accomplished by a simple preference of a run of purine bases or a run of pyrimidine bases since alternating copolymers like $(A\text{–}U)_n$ or $(I\text{–}C)_n$ are interferon inducers. Since $(A)_n \cdot (U)_n$, $(I)_n \cdot (C)_n$, $(c^7I)_n \cdot (br^5C)_n$, $(s^2C)_n \cdot (I)_n$, $(I)_n \cdot (br^5C)_n$ and $(A)_n \cdot (rT)_n$ are all active inducers, purine sites N^7, HN^6, O^6, HN^2 as well as pyrimidine sites O^4, HN^4, C^5, and probably O^2 can be eliminated as potential recognition sites leaving only purine N^3, N^1 and C^8 in addition to pyrimidine C^6 and N^3 as potential recognition sites. Purine N^1 and pyrimidine N^3 would become available only after helix denaturation, an unlikely event for reasons previously outlined. Recognition could not be based on purine N^3 alone since $(c^7A)_n$, $(L)_n$ and $(X)_n$ derived duplexes are inactive inducers; similar reasoning can be invoked to dismiss the possibility of recognition through purine C^8 or pyrimidine C^6. Mechanisms invoking combinations of purine N^3 with either purine C^8 or pyrimidine C^6 would require involvement of both the major and minor grooves in the recognition, a thermodynamically unlikely process. It would appear that at least for the case of

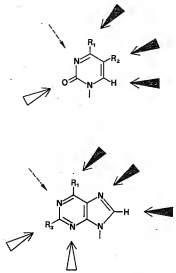


FIG. 11. Potential recognition sites on purine or pyrimidine bases. All indicated sites would be available for recognition for the case of a denatured nucleic acid. The symbol ⇛ denotes those positions accessible from the major groove of a double-helical nucleic acid; ⇨ denotes accessibility from the minor groove of a double-helical nucleic acid; → denotes those sites that would be inaccessible from either groove unless the double-helical nucleic acid were at least partially denatured. For ease of illustration, rare tautomeric forms of uracil, guanine and hypothanine are implied.

interferon induction by synthetic polynucleotides, the independence of one strand over the other would be difficult to rationalize on a mechanistic basis.

Before proceeding to discussion of another theory of the origin of the structural dependence of interferon induction by polynucleotides, we digress to review nucleic acid conformation. Several excellent reviews of this topic are available (Arnott, 1970; Arnott *et al.*, 1975; Sundaralingam, 1975). Although many parameters are required to totally describe a helical polynucleotide, because of their rigidity, significant variations are found in only a few. Those that will be considered are: base-pair twist, base-pair tilt, displacement of the base-pairs from the helical axis (D), axial rise per residue (h) and the related pitch (distance of one full helical turn), and pucker of the sugar ring (as defined in Figs 12 and 13).

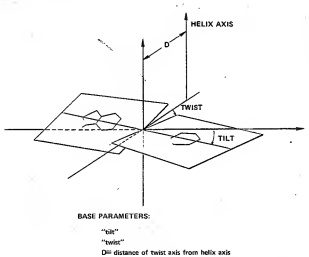
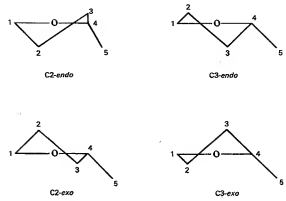


FIG. 12. Definition of the base-parameters of a helical nucleic acid; namely, tilt (a measure of the displacement of the base-pairs with respect to a perpendicular to the helix axis, twist (a measure of the relation of the plane of one base to the plane of the other member of the base-pair) and D (a measure of the displacement of the base-pair from the helix axis). After Arnott, 1970 (with permission).



Projections parallel to C1-05-C4 showing the four modes of sugar puckering

FIG. 13. The various possible extremes of conformation that may be assumed by the ribose ring in a polynucleotide.

The conformation of a helical nucleic acid depends on the composition and sequence of the nucleotide building blocks (and to some extent on the helical environment). The support for this statement comes from X-ray fiber-diffraction studies on nucleic acids as reviewed by Arnott *et al.* (1975) and illustrated in Table 15. Inspection of this data shows that the conformational parameters change significantly as the nature and sequence of the nucleic acid bases change. Furthermore, changes in one helical parameter are often reflected in changes in another parameter; for instance, an empirical correlation between tilt of the base-pairs and h (axial rise/residue) has been established (Arnott *et al.*, 1975), and a good correlation exists relating the sugar ring conformation to D and tilt (Arnott *et al.*, 1975).

The implications that such alterations in nucleic acid conformation have for protein–nucleic recognition are enormous. Consider a protein receptor designed to specifically recognize the helical conformation of dsRNA (always of the A family of nucleic acids). Inspection of Fig. 14 and Table 15 suggest that such a protein could not effectively recognize native DNA of the B family of conformations. Let us consider a hypothetical example. A protein with two conformationally fixed lysine residues oriented to interact electrostatically with two polynucleotide phosphate groups 31 Å apart would not interact well with such groups in dsDNA where the corresponding helically oriented phosphates would be 34 Å apart, since the attraction is related to the square of the distance between the charges. Protein–nucleic acid recognition processes will be much more complex than this simple system, involving cooperative interaction through both electrostatic (including hydrogen-bonding) and hydrophobic

TABLE 15. *Some Comparisons of the Conformations of Different Families of Nucleic Acids*

Nucleic acid family	Helix pitch	h	Base-pairs/turn	Tilt	D	Sugar conformation
A–RNA						
e.g. $(A)_n$·$(U)_n$						
$(I)_n$·$(C)_n$	30.9 Å	2.82 Å	11	17.4°	4 Å	C3-endo
A'-RNA						
e.g. $(I)_n$·$(dC)_n$	36.2 Å	3.0 Å	12	12.5°	4 Å	C3-endo
A–DNA						
transition of native DNA	28 Å	2.56 Å	11	20.2°	4.2 Å	C3-endo
B–DNA						
e.g. native DNA						
$(dA)_n$·$(dT)_n$	34 Å	3.37 Å	10	6.3°	0 Å	C3-exo
D–DNA						
e.g. $(dA–dT)_n$·$(dA–dT)_n$	24 Å	3.03 Å	8	16.4°	−1.8 Å	C3-exo

Values gathered from Arnott *et al.*, 1973, 1974b and references therein.
Helix parameters are defined in the text and in Figs 12 and 13.

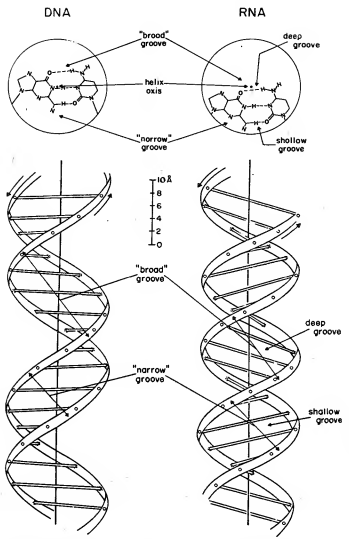


FIG. 14. Illustrations of the B–DNA and B–RNA double-helices as viewed either down the helix axis (top) or perpendicular to the helix axis (bottom). The 'narrow' or minor groove and 'broad' or major groove of the molecules are shown. Also the 'deep' groove and 'shallow' groove in B–RNA are indicated. (Reproduced with permission from Sundaralingam, 1975, p. 506).

forces occurring minimally over a region of several nucleotide pairs and several amino acids. A conformational change of the nucleic acid would evoke dramatic alterations in the free energy of binding.

On the basis of these considerations, coupled with the experimental findings outlined in the previous sections, the hypothesis has been presented that interferon induction is dependent on the recognition of a particular spatial and steric organization of a double-stranded nucleic acid (Johnston *et al.*, 1975; Torrence *et al.*, 1975b; Torrence and Witkop, 1976). Two studies relate directly to this hypothesis. Considering the established facts that nucleic acid interferon inducers are double-stranded and that their inducing ability is dependent on both the ribose–phosphate backbone on the exterior of the helix and the base in the interior of the helix, Johnston *et al.* (1975) raised the question: can any other protein receptor of nucleic acids 'recognize' the apparent differences among ds-nucleic acids in the same way as the interferon induction process does? Experimentally induced antibodies to dsRNA, because they have specificities to conformational aspects of structure rather than to the individual base or ribose components of nucleic acids (Stollar, 1973, 1975) were used as a model dsRNA–protein recognition system. Anti-$(A)_n \cdot (U)_n$ sera were ob-

tained by immunizing rabbits with $(A)_n \cdot (U)_n$-methylated bovine serum albumin complex followed by purification of the antisera by precipitation with $(I)_n \cdot (C)_n$ and dissociation to give antibodies directed specifically against dsRNA. The interaction of this antibody with modified dsRNAs was then studied by the technique of quantitative complement fixation (c'F).

Two types of changes occurred in the c'F curves: (1) for a given quantity of antibody, all modifications resulted in a decrease in the amount of complement fixed, as shown by vertical shifts of the c'F curves; (2) In some instances, the amount of nucleic acid needed to reach the maximum c'F was constant. With other duplexes, increased quantities of nucleic acid were required to achieve maximum c'F, and this resulted in horizontal shifts of the curves. The vertical shifts in the c'F curves were determined quantitatively and employed to obtain titers and indices of dissimilarity. Titers are defined simply as the inverse of the antibody dilution required to give a curve having a maximum of 50 per cent c'F. Index of dissimilarity is defined as the titer for $(A)_n \cdot (U)_n$ divided by the titer for modified dsRNAs.

Table 16 presents these two experimentally derived values (i.e. titers and indices of dissimilarity) as well as an indication of the quantity of hapten needed to obtain maximum c'F. For the base modified dsRNAs, the order of reactivity in the $(I)_n \cdot (C)_n$ series was: $(I)_n \cdot (C)_n > (c^7I)_n \cdot (C)_n > (c^7I)_n \cdot (br^5C)_n \gg (L)_n \cdot (C)_n > (L)_n \cdot (br^5C)_n$. In the $(A)_n \cdot (U)_n$ series the order of reactivity was: $(A)_n \cdot (U)_n > (A)_n \cdot (rT)_n > (A)_n \cdot (br^5U)_n > (c^7A)_n \cdot (rT)_n > (c^7A)_n \cdot (br^5U)_n > (c^7A)_n \cdot (U)_n$. Of all the 2'-modified polymers, including $(A)_n \cdot (Um)_n$, $(Am)_n \cdot (U)_n$, $(Am)_n \cdot (Um)_n$, $(I)_n \cdot (Cm)_n$, $(A)_n \cdot (dUfl)_n$, $(Ae)_n \cdot (U)_n$, $(A)_n \cdot (dUz)_n$ and $(I)_n \cdot (dCcl)_n$, only two showed any reaction; viz., $(A)_n \cdot (dUz)_n$ and $(I)_n \cdot (dCcl)_n$, but these required a high antibody concentration as well as a high hapten concentration.

This study showed, in accord with previous suggestions (Stollar, 1975), that the specificity of dsRNA antibodies involves recognition of the pentose-phosphate backbone on the outside of the helix, and both strands of the double-helix contribute to the formation of a single antigenic determinant. The presence of the ribose-phosphate backbone was not sufficient to insure recognition by the antibody, since all single-stranded polymers were unreactive; however, the ribose must play an important role, since nearly all 2'-modifications resulted in a drastic decrease in serological reactivity. Thus, a particular conformation of the ribose could serve directly as a binding site or as a major contributor to the overall conformation of the ribose–phosphate backbone. Since modifications of the sugar in just one chain had a profound effect, and since the weakly reactive $(L)_n$ duplexes and the unreactive $(G)_n \cdot (C)_n$ duplexes contain unmodified ribose in both strands, the backbones of the two strands presumably must be in some specific relation to one another. Inasmuch as individual bases are not

TABLE 16. *Reactivities of Modified Double-stranded Polynucleotides with Anti-*$(A)_n \cdot (U)_n$ *Antibody**

Inducers of interferon	Relative antigen conc. to obtain max. c'F	Titer	Index of dissimilarity
$(A)_n \cdot (U)_n$	1	680	1.0
$(I)_n \cdot (C)_n$	1	450	1.5
$(A)_n \cdot (rT)_n$	1	350	1.9
$(I)_n \cdot (br^5C)_n$	3	170	4.0
$(c^7I)_n \cdot (C)_n$	9	240	2.8
$(c^7I)_n \cdot (br^5C)_n$	27	110	6.2
Non-inducers:			
$(c^7A)_n \cdot (U)_n$	3	125	5.0
$(c^7A)_n \cdot (rT)_n$	3	180	3.8
$(c^7A)_n \cdot (br^5U)_n$	9	160	4.2
$(A)_n \cdot (br^5U)_n$	3	270	2.5
$(L)_n \cdot (C)_n$	27	40	17
$(L)_n \cdot (br^5C)_n$	27	30	22
$(I)_n \cdot (dCcl)_n$	27	120	5.7
$(A)_n \cdot (dUz)_n$	1	100–130	5.2–6.8

* All single-strands were inactive even with undiluted antisera.
See text for definitions. Data taken from Johnston *et al.* (1975).

recognized by antibody against $(A)_n \cdot (U)_n$, serological changes accompanying base modification may also reflect changes in helical conformation.

Table 17 compares these two nucleic acid receptor systems, antibodies against dsRNA and interferon induction by nucleic acids. These similarities provide strong circumstantial evidence in support of the hypothesis that one of the early steps in the interferon induction process involves recognition (probably by a protein*) of a particular spatial and steric configuration of the nucleic acid double-helix.

A 1:1 correspondence between reactivity of antibody and interferon-inducing ability is not to be expected, since as illustrated in Fig. 15, different recognition mechanisms could be involved and would therefore differ in their sensitivity to a given structural change.

A second study (Bobst *et al.*, 1976) was directed at resolving the difference in interferon-inducing capacity of the $(c^7A)_n$ duplexes and the $(c^7I)_n$ duplexes, the former group being unable to induce interferon and the latter group being comparable in activity to $(I)_n \cdot (C)_n$. Ultraviolet and circular dichroism (CD) characteristics of these analogs were compared to the corresponding $(A)_n$ duplexes and $(I)_n$ duplexes. Interpretation of the changes in CD relied upon the earlier theoretical studies of Studdert and Davis (1974) and, in addition, required the assumption that since all investigated duplexes were ribopolymers, they would possess the value of D (displacement of base-pair from helical axis) characteristic of the A family of nucleic acids. The spectra were interpreted as showing that pyrimidine-5 substitution by bromine or methyl changes the conformation of both $(c^7I)_n$ and $(c^7A)_n$ duplexes (compared to $(A)_n$ and $(I)_n$ duplexes) in a similar manner, if at all. To the contrary, replacement of purine N^7 by CH had opposing effects depending on whether the change was effected in the $(A)_n$ series or the $(I)_n$ series. The modification $(A)_n \rightarrow (c^7A)_n$ resulted in an increase in the positive base tilt whereas the change $(I)_n \rightarrow (c^7I)_n$ caused a decrease. If the correlation of Arnott *et al.* (1975) is followed, this would imply that h (axial rise/residue) is decreased relative to $(I)_n \cdot (C)_n$ or $(A)_n \cdot (U)_n$ in the $(c^7A)_n$ complexes and increased in the $(c^7I)_n$ complexes. From this study, it is, of course, not possible to define completely all the conformational parameters of the helices, and it

TABLE 17. *Comparisons of two Different Nucleic Acid Receptor Systems*

Nucleic acid	Antibody to dsRNA	Interferon induction[a]
Single-stranded RNA or DNA	No reactivity[b,c]	No induction
Native or synthetic dsDNA	No reactivity[b,c]	No induction
RNA–DNA hybrids	Some limited reactivity but at significantly altered antisera concentration[b,c]	No induction
Triple-helical nucleic acids	Reactive, but quantitatively shifted. May be true cross-reactivity or may be due to small quantities of dsRNA regions in triplex[b,c]	No induction but some triplexes may reduce the titer of active inducers
Alterations in base content or sequence in dsRNAs	E.g. antisera to $(A)_n \cdot (U)_n$ reacts equally well with $(A)_n \cdot (U)_n$, $(I)_n \cdot (C)_n$, reovirus dsRNA and viral replicative forms[b,c]	E.g. $(A)_n \cdot (U)_n$, $(I)_n \cdot (C)_n$ reovirus dsRNA and viral replicative forms are all potent inducers
dsRNA with modified 2′-OH groups in one or both strands	Reactivity abolished or significantly altered[d]	No induction
dsRNA based on $(L)_n$	Large decrease in reactivity[d]	No induction
dsRNA based on $(c^7A)_n$	Quantitative shifts in reactivity[d]	No induction
dsRNA based on $(c^7I)_n$	Quantitative shifts in reactivity[d]	Effective inducers

[a] See earlier sections in this review for references.
[b] Stollar, 1973.
[c] Stollar, 1975.
[d] Johnston *et al.*, 1975.

* Colby and Chamberlin (1969) introduced the hypothesis that the interferon inducer receptor may be a protein.

HIGHLY SIMPLIFIED REPRESENTATION OF THE INTERACTION OF HYPOTHETICAL RECEPTORS WITH DOUBLE-STRANDED NUCLEIC ACID

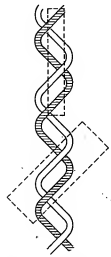


FIG. 15. Highly schematic hypothetical representation of two different modes of recognition that could be used by a receptor for dsRNA.

is not possible to specify which parameter change(s) is (are) responsible for the difference in interferon-inducing behavior. The study did show, however, that an alteration of conformation accompanies the loss of interferon inducing ability when proceeding from the $(c^7I)_n$ duplexes to the $(c^7A)_n$ duplexes.

It is a simple matter to relate the lack of interferon-inducing ability of other double-helical nucleic acids to their altered conformation relative to active inducers like $(I)_n \cdot (C)_n$. For instance, the homopolymer double-helix, $(s^2U)_n$, does not induce interferon even though it has a high T_m (62.5°) (Reuss *et al.*, 1976). This double-helix has a markedly altered conformation: there exists no symmetry element as with the DNA or RNA double-helices. Thus, the two chains have different conformations, and pair according to an entirely different mode of base hydrogen-bonding (Mazumdar *et al.*, 1975). Another example is that of the inactive $(I)_n \cdot (C)_n$ analogs, $(L)_n \cdot (C)_n$ and $(L)_n \cdot (br^2C)_n$. Although no information from CD or X-ray diffraction is yet available on the conformation of these complexes, it is reasonable to expect that they will differ from $(I)_n \cdot (C)_n$. The radically altered heterocyclic ring of the $(L)_n$ complexes will likely change base-stacking interactions in the polymers, and the longer C–C glycoside bond may change the base-sugar interactions and/or sugar conformation (e.g. Prusiner *et al.*, 1973).

According to the arguments presented above, it is possible that the inactivity of 2′-modified polynucleotides may have different origins. This possibility is examined with the aid of Fig. 16, which also serves to illustrate how interferon induction may be influenced by the configuration of the ribose–phosphate backbone. Consider that the receptor for the dsRNA possesses some site that can perceive the 2′-OH group (as originally suggested by Colby (1971)). Substitution of hydrogen for the 2′-OH group (as in DNA) alters the conformation of the helix from the A family (C3-endo) to the B family (C3-exo) so that the 2′-substituent is no longer in proximity to the protein 'groove' that binds the 2′-OH group. Even if the DNA attained the A form (as in DNA–RNA hybrids), the 2′-hydrogen could not bind to the receptor 'groove' because of the obvious alterations in steric bulk, hydrogen-bonding capabilities, etc. Either way, a considerable loss of protein-nucleic acid binding would occur. Substitution of 2′-OCH_3 for 2′-OH would be equally disastrous for the nucleic acid-receptor in-

ROLE OF THE 2′–OH GROUP IN NUCLEIC ACID-RECEPTOR INTERACTIONS

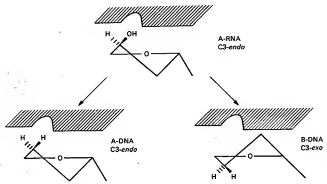


FIG. 16. A presentation of one hypothesis as to why 2′-modifications of dsRNAs lead to inactive interferon inducers. As discussed in the text, modification of the ribose 2′-hydroxyl may effect a loss of binding to this putative receptor either because of a conformation 'flip' of the ribose or because of a marked change of the substituent's affinity for the site that normally accommodates the hydroxyl.

teraction. Introduction of the OCH_3 group could either change the conformation of the sugar to C3-exo, or it could prevent binding simply by its increased steric bulk *even if there were no alterations in conformation.* The former possibility seems rather unlikely since RNA double-helices themselves are not inclined to adopt the B conformation due to the unfavorable steric compression that would result (e.g. Arnott *et al.*, 1975). Some 2′-modified dsRNAs may not induce interferon because they have an altered ribose-phosphate backbone conformation (as is the case for DNA): others do not induce because the 2′-substituent may fail to effectively mimic the 2′-hydroxyl group in its physical properties.

The hypothesis outlined above, that interferon induction is dependent on the recognition of a particular spatial and steric configuration of the double-helix and is not dependent on the recognition of individual bases, need not be considered inconsistent with the mechanism of other nucleic acid-protein recognitions. In one of the most intensively studied systems, the *Escherichia coli lac* repressor, several mechanisms have been considered for the specificity of the *lac* repressor for the *lac* operator. These include: (1) base recognition of fully or partially denatured operator; (2) base recognition through the major groove of the double-helix; (3) base recognition through the minor groove of the double-helix, (4) recognition of a specific conformation of the deoxyribosephosphate backbone; and (5) combinations of the above. Richmond and Steitz (1976) have recently presented excellent evidence against possibilities (1) and (2) above. They placed various mercaptan ligands in the major groove of the synthetic DNA, $(dA\text{-}dU)_n$, via covalently bound mercury in the 5-position of the pyrimidine. Even though these ligands virtually packed the major groove of DNA, the modified DNAs still had a high affinity for the repressor protein. Furthermore, cross-linking the two strands of the modified helix with dithiothreitol, so that no denaturation could occur, also gave rise to a DNA with high affinity for the repressor.

11. ADDITIONAL EFFECTS OF DOUBLE-STRANDED RNA'S ON CELLULAR METABOLISM

In addition to their well-documented interferon-inducing activity, double-stranded RNAs, such as $(I)_n \cdot (C)_n$, exert a wide variety of biological effects. Quite often these effects emerge not only at the level of the whole organism but even at the cellular level.

One of the most striking properties of double-stranded RNAs is their adjuvant effect on both cellular and humoral immune responses. This immunoadjuvant effect is

most characteristically reflected by the stimulatory activity of both $(I)_n \cdot (C)_n$ and $(A) \cdot (U)_n$ on DNA synthesis (as measured by [^{3}H]thymidine incorporation) of mouse spleen lymphocytes (Dean *et al.*, 1972; Rühl *et al.*, 1974; Woods *et al.*, 1974; Ts'o *et al.*, 1976) and human peripheral blood lymphocytes activated by PPD (purified protein derivative) or other antigens (Friedman *et al.*, 1969; Graziano *et al.*, 1974). However, DNA synthesis of lymphocytes which have been stimulated by phytohemagglutinin is suppressed by $(I)_n \cdot (C)_n$ and $(A)_n \cdot (U)_n$ (Friedman *et al.*, 1969; Badger *et al.*, 1972). The latter effect may well be mediated by interferon since interferon itself has been shown to curtail DNA synthesis in lymphocytes stimulated by phytohemagglutinin, concanavalin A and other mitogenic stimuli (Lindahl-Magnusson *et al.*, 1972; Rozee *et al.*, 1973; Blomgren *et al.*, 1974).

That the stimulatory effect of $(I)_n \cdot (C)_n$ on [^{3}H]thymidine incorporation of the murine splenic cells, as noted above, can actually be interpreted in terms of increased DNA synthesis is suggested by the observation that blast-cell transformation closely paralleled [^{3}H]thymidine incorporation (Dean *et al.*, 1972).

In conjunction with its mitogenic effect on lymphocytes, $(I)_n \cdot (C)_n$ has also been shown to stimulate colony formation by mouse and human hemopoietic (bone marrow) cells when these cells are plated *in vitro* (McNeill, 1971; Mangalik *et al.*, 1975).

$(I)_n \cdot (C)_n$ has also been reported to increase [^{3}H]thymidine incorporation into DNA of continuous cell lines such as HeLa and mouse L cells (Badger *et al.*, 1972); however, this increment of thymidine incorporation was directly related to a cytotoxic effect of $(I)_n \cdot (C)_n$ and could therefore be interpreted as the consequence of DNA damage and an attempt at DNA repair. When studied in more rigorous conditions (synchronized HeLa cells), $(I)_n \cdot (C)_n$ actually inhibited DNA synthesis (Teng *et al.*, 1973). In these synchronized cells, inhibition of DNA synthesis by $(I)_n \cdot (C)_n$ was confined to a specific time in the cell cycle (late G_1 and early S phases).

In tumor-bearing mice (e.g. C57 black mice), $(I)_n \cdot (C)_n$ may inhibit RNA and protein synthesis in tumor tissue (e.g. J96132 reticulum cell sarcoma) while increasing RNA or protein synthesis in normal tissues (Levy and Riley, 1970). In other strains of mice, however, macromolecular synthesis in normal organs is equally well inhibited as macromolecular synthesis in tumor tissue.

$(I)_n \cdot (C)_n$ has also been reported to suppress isoproterenol-stimulated DNA synthesis in the salivary glands of mice (Serota and Baserga, 1970). This effect was thought to be relatively selective and, to the best of our knowledge, it has never been demonstrated with exogenous interferon. Mitosis of liver cells in partially hepatectomized mice and proliferation of allogeneic spleen cells or syngeneic bone marrow cells in X-irradiated mice are also inhibited by interferon inducers such as $(I)_n \cdot (C)_n$, but these effects are readily duplicated by exogenous interferon (Jahiel *et al.*, 1971; Cerottini *et al.*, 1973; Frayssinet *et al.*, 1973).

Despite its stimulatory effects on hemopoietic cell colony formation *in vitro*, $(I)_n \cdot (C)_n$ severely afflicts spleen and bone marrow colony forming cells *in vivo* (mice) (Jullien and De Maeyer-Guignard, 1971). Repeated injections of $(I)_n \cdot (C)_n$ almost totally deplete the bone marrow of its colony forming units (Martelly and Jullien, 1974). The toxic effect of $(I)_n \cdot (C)_n$ on mouse hemopoietic stem cells does not seem mediated by interferon, since Newcastle disease virus, inoculated at a dose which induced comparable levels of circulating interferon, failed to affect colony formation in the bone marrow (Jullien and De Maeyer-Guignard, 1971).

Various other adverse effects have been observed with $(I)_n \cdot (C)_n$ in the whole animal. As reviewed previously (Carter and De Clercq, 1974), these effects include a local Shwartzman phenomenon, pyrogenicity, embryotoxicity, and ocular toxicity (in the rabbit), leukopenia, thymic atrophy, spleen hypoplasia and an accelerated onset of autoimmune disease (in the mouse), and general edema and hemorrhages due to destruction of the endothelial cells of the small vessels (in the chicken).

The question arises as to the molecular basis of each of these toxic manifestations. Although different possibilities could be entertained, there is one particular aspect in

the molecular action of double-stranded RNA that will deserve our further attention; namely, its inhibitory effect on protein synthesis. A large variety of double-stranded RNA molecules—nearly as large as that known to induce interferon—has been reported to inhibit protein synthesis in rabbit reticulocyte lysates (Hunter *et al.*, 1975). Maximum inhibition was observed in the concentration range of 0.01–0.1 μg/ml, which corresponds well to the minimum effective dose range inducing interferon and resistance to virus infection in the most sensitive cell cultures (e.g. primary rabbit kidney cells). As noted for interferon induction, the ability of dsRNAs to inhibit globin synthesis in reticulocyte lysates depends on a properly matched dsRNA segment of at least fifty base pairs (in length (~4 S)) (Hunter *et al.*, 1975).

Puzzling features of the inhibitory activity of dsRNAs on protein synthesis are that: (1) at supra-optimal concentrations ($\geq$10 μg/ml) dsRNAs fail to inhibit protein synthesis in reticulocyte lysates (Hunter *et al.*, 1975); and (2) that potent inhibition of protein synthesis by dsRNA is almost a peculiarity of cell-free systems prepared from reticulocytes (Table 18). Substantially higher concentrations of dsRNA are required to demonstrate inhibition of protein synthesis in cell-free extracts other than reticulocyte lysates, e.g. Krebs II ascites cell extracts. The lowered sensitivity of the Krebs II ascites cell extract to dsRNA is apparently due to the presence in the extract of a nuclease that readily digests dsRNAs (Robertson and Mathews, 1973). There are a number of other cell-free systems which have not been found sensitive to inhibition by dsRNAs: e.g. endogenous protein synthesis in chick embryo lysates (Shenk and Stollar, 1972), poly (U)-directed polyphenylalanine synthesis in both chick embryo and rabbit reticulocyte extracts (Shenk and Stollar, 1972), protein synthesis in wheat germ extracts programmed with various mRNAs (Reijnders *et al.*, 1975; Grill *et al.*, 1976). The failure of dsRNA to suppress protein synthesis in the wheat germ system is not due to the presence of nucleolytic activity in the wheat germ extract (Grill *et al.*, 1976). Although Chao *et al.* (1971) reported that $(I)_n \cdot (C)_n$ may inhibit f2 RNA directed protein synthesis in *E. coli* extract by virtue of a direct interaction between $(I)_n \cdot (C)_n$ and f2 RNA, bacterial protein synthesis is generally considered resistant to inhibition by dsRNA.

Inactivation of the initiation factor IF-3 would account for the inhibitory activity of

TABLE 18. *Inhibition of Protein Synthesis by Double-stranded RNAs in Cell-free Systems*

Cell-free system	Protein synthesis directed by:	Double-stranded RNA inhibiting protein synthesis	Leading references
Rabbit reticulocyte lysate	Endogenous mRNA	Poliovirus RNA (replicative form)	Hunt and Ehrenfeld, 1971 Ehrenfeld and Hunt, 1971
		Sindbis virus RNA (replicative form)	Shenk and Stollar, 1972
		Cowpea mosaic virus RNA (replicative form)	Reijnders *et al.*, 1975
		dsRNA extracted from nuclei of HeLa cells	Bases and Kaplan, 1973
		Human leukemic blast cells	Torelli *et al.*, 1975
		Reovirus dsRNA	Hunter *et al.*, 1975 Grill *et al.*, 1976
		$(I)_n \cdot (C)_n$	Shenk and Stollar, 1972 Hunter *et al.*, 1975 Grill *et al.*, 1976
		Other dsRNAs	Hunter *et al.*, 1975
Krebs II ascites cell extract	Encephalomyocarditis virus mRNA	Penicillium chrysogenum dsRNA F_2 RNA (replicative form) Reovirus dsRNA	Robertson and Mathews, 1973
E. coli extract	F_2 virus mRNA	$(I)_n \cdot (C)_n$*	Chao *et al.*, 1971
Mouse L cell extract	Reovirus mRNA	Reovirus dsRNA	Graziadei and Lengyel, 1972
HeLa cell extract Poliovirus-infected HeLa cell extract	Endogenous mRNA	Poliovirus RNA (replicative form)	Celma and Ehrenfeld, 1974

* Inhibitory effect attributed to a direct interaction of $(I)_n \cdot (C)_n$ with f_2 RNA.

dsRNA on protein synthesis in eukaryote systems (Kaempfer and Kaufman, 1973). As a consequence, no complex is formed between the initiator tRNA (formylmethionyl-tRNA) and the native 40 S ribosomal subunits (Darnbrough *et al.*, 1973), and the translation process is shut off. Although the exact mechanism by which dsRNA inactivates the initiation factor IF-3 has not been elucidated (Kaempfer, 1974), it is clearly not the result of a simple stoichiometric interaction between IF-3 and the double-stranded RNA (Hunter *et al.*, 1975). As summarized in Table 19, several events are associated with the inhibition of protein synthesis by dsRNA in cell-free systems. The relationship between these different effects and the common cause of all these effects remain to be established.

Double-stranded RNAs not only inhibit protein synthesis in cell-free systems, but they also suppress protein synthesis in intact cells: e.g. the addition of bovine enterovirus double-stranded RNA to intact cells in culture results in the cessation of host cell protein synthesis, rapidly followed by an extensive cell destruction (Cordell-Stewart and Taylor, 1971, 1973a, 1973b). The decline in host protein synthesis generally observed in cells infected with lytic viruses (e.g. poliovirus) would seem related to the formation of the replicative dsRNA intermediate in these cells. Accumulation of this dsRNA in the infected cell would ultimately lead to a complete shut-off of cellular protein synthesis and death of the cell.

The toxicity of dsRNA, whether synthesized within the cell during the replicative cycle of the virus or added exogenously to the cells, may be further potentiated by previous exposure of the cells to interferon. This toxicity-enhancing effect of interferon is most dramatic in mouse L cells (Stewart *et al.*, 1972a, 1973a; De Clercq and De Somer, 1975) and strictly limited to double-stranded RNAs. Interferon-treated L cells exhibit no enhanced susceptibility to the toxicity of single-stranded RNAs, double-stranded DNAs, endotoxin, cycloheximide, actinomycin D, diphtheria toxin, cholera toxin, snake venom and other materials (Stewart *et al.*, 1973a). A possible explanation for the enhanced toxicity of dsRNA in the interferon-treated cell has been provided by Kerr *et al.* (1974, 1976) who showed a marked increase in the inhibitory effect of dsRNA on protein synthesis (programmed by encephalomyocarditis virus mRNA) in extracts prepared from interferon-treated L cells as compared to extracts prepared from untreated L cells. In other studies, Roberts *et al.* (1976a) attributed the synergistic inhibitory action of dsRNA and interferon to the activation by dsRNA of an inhibitor present in the postribosomal supernatant fraction (cell sap) of the interferon-treated cells. The activation step appeared to be associated with phosphorylation of some polypeptides (not the inhibitor itself) by a specific dsRNA-dependent protein kinase (Roberts *et al.*, 1976b).

Quite similar observations have been reported by Lengyel and his colleagues (Brown *et al.*, 1976; Sen *et al.*, 1976; Lebleu *et al.*, 1976). In their cell-free system (extracts of Ehrlich ascites tumor cells), dsRNA inhibited protein synthesis (directed

TABLE 19. *Events Associated with Inhibition of Protein Synthesis by Double-stranded RNAs*

I. In cell-free systems prepared from normal cells (rabbit reticulocytes)
   - Inactivation of an initiation factor (Kaempfer and Kaufman, 1973; Kaempfer, 1974)
   - Inhibition of complex formation between formylmethionyl-tRNA and the 40 S ribosomal subunit (Darnbrough *et al.*, 1973)
   - Activation of an inhibitor (Hunter *et al.*, 1975)
   - Protein kinase activity and phosphorylation of an initiation factor (P. Farrell, K. Balkow, T. Hunt and R. J. Jackson: personal communication in Roberts *et al.*, 1976b)

II. In cell-free systems prepared from interferon-treated cells
   - (1°) *Mouse L cells* (protein synthesis directed by encephalomyocarditis virus mRNA)
     - Activation of an inhibitor (Kerr *et al.*, 1974; Roberts *et al.*, 1976a)
     - Increased rate of degradation of mRNA (Kerr *et al.*, 1976)
     - Protein kinase activity (Roberts *et al.*, 1976b)
   - (2°) *Ehrlich ascites tumor cells* (protein synthesis directed by reovirus mRNA)
     - Increased rate of degradation of mRNA, as a consequence of increased susceptibility of the mRNA to endonuclease (Brown *et al.*, 1976; Sen *et al.*, 1976)
     - Phosphorylation of proteins P_1 and P_2 (Lebleu *et al.*, 1976)

by reovirus mRNA) by virtue of an enhanced degradation of the mRNA. The enhanced RNA degradation was particularly apparent in extracts prepared from interferon-treated cells. It was attributed to an increased endonuclease activity. The endonuclease may have been activated by phosphorylation, since dsRNA was found to promote the phosphorylation of at least two proteins in extracts from interferon-treated Ehrlich ascites tumor cells (Lebleu *et al.*, 1976).

The different events occurring upon addition of dsRNA to cell-free extracts prepared from interferon-treated cells are summarized in Table 19. How these effects might be linked to each other, and how they may be related to the effects of dsRNA on the intact functioning cell remains to be established.

12. HYPORESPONSIVENESS TO INTERFERON PRODUCTION

Interferon production is generally followed by a period during which restimulation with inducer gives rise to little, if any, interferon. This phenomenon occurs both *in vitro* and *in vivo* (though not necessarily by the same mechanism), and has been variously termed 'hyporesponsiveness', 'hyporeactivity', 'refractoriness', 'tolerance' and 'resistance'. It was described first by Ho and Kono (1965a) and Youngner and Stinebring (1965). Together with toxicity and immunogenic potential, hyporesponsiveness stands as a major impedance to the ultimate clinical application of interferon inducers: for instance, repeated application of $(I)_n \cdot (C)_n$ to the eyes of rabbits led to a loss of detectable interferon production coupled with susceptibility to infection with herpes simplex virus (Centifanto *et al.*, 1970). Hyporeactivity to interferon induction and hyporeactivity to protection against virus infection closely parallel each other in some conditions (De Clercq *et al.*, 1970d; De Clercq, 1972) but not in others (Sharpe *et al.*, 1971). Possibly, this potential obstacle of hyporesponsiveness could be overcome by careful control of the dosage and frequency of administration (Baron *et al.*, 1970; Ho *et al.*, 1970; DuBuy *et al.*, 1970; Buckler *et al.*, 1971); however, the possible limitation of the latter approach (to prophylaxis alone) is suggested by the findings that several viral infections (lymphocytic choriomeningitis virus, cytomegalovirus, Friend leukemia virus, encephalomyocarditis virus) in mice led to a reduced ability to produce interferon (Stringfellow and Glasgow., 1972a; DeMaeyer-Guignard, 1972; Hoterman and Havell, 1970; Osburn and Medearis, 1967).

As reviewed by Ho (1973), the development of hyporesponsiveness *in vivo* consistently occurs when repeated injections of the same inducer are administered (contrast with Sharpe *et al.*, 1971). When two different inducers are administered, development of the hyporesponsive state depends on the nature and dose of the inducer as well as the animal species involved. Although the induction of a humoral factor has been frequently considered as a possible explanation for *in vivo* hyporesponsiveness (Paucker and Boxaca, 1967; Youngner and Stinebring, 1965; Ho *et al.*, 1965a; Borden and Murphy, 1971), until recently, no direct evidence for such a factor (with the exception of endotoxin tolerance (Ho *et al.*, 1965a)) has been provided. At least in the case of hyporesponsiveness promoted by viral infection, there does appear to be a transferable protein factor, physicochemically similar to interferon, that may be at least partly responsible for the relative inability of encephalomyocarditis virus infected mice to respond to a variety of inducers (Stringfellow and Glasgow, 1972a, 1974; Stringfellow, 1975).

As far as *in vitro* hyporesponsiveness is concerned, the hypothesis has been advanced (Vilček, 1970b; Billiau, 1970; Vilček *et al.*, 1972; Breinig *et al.*, 1975; Kohase and Vilček, 1977) that a relationship exists among three characteristics of interferon production; namely, hyporesponsiveness, the paradoxical enhancement of interferon synthesis by metabolic inhibitors ('superinduction') and the early shut-off of interferon production. The common link may be the formation of a labile repressor protein which exerts negative control on interferon synthesis. Several studies have shown that hyporesponsiveness is not directly correlated with the presence or production of interferon or with the development of the antiviral state (Billiau, 1970; Margolis *et al.*,

1972; Borden and Murphy, 1971; Breinig *et al.*, 1975). Vilček *et al.* (1972) found that while rabbit kidney cells stimulated with $(I)_n \cdot (C)_n$ responded with an early shut-off of interferon synthesis, cells treated with $(I)_n \cdot (C)_n$ in the presence of DEAE-dextran gave a protracted interferon response which, in contrast to the former, was not enhanced by superinduction procedures. Cells that were made hyporesponsive to $(I)_n \cdot (C)_n$ alone could be efficiently restimulated with $(I)_n \cdot (C)_n$ + DEAE-dextran. However, Rousset (1974) could find no evidence to distinguish the substance responsible for hyporesponsiveness from interferon itself. Rousset suggested that the hyporesponsive state might arise *via* an interferon induced protein that could act as a feed-back inhibitor of interferon synthesis (see also Chany and Vignal, 1970; Youngner and Hallum, 1969; Bausek and Merigan, 1970).

Several studies suggest that hyporesponsiveness to interferon production results from a refractoriness-inducing-principle (RIP) and that interferon and RIP are different biological entities and not polymorphic functions of the same molecule:

(a) To elicit the refractory state, a significantly longer contact period of the cell with interferon is needed as compared to the time of contact required for development of the antiviral state (Paucker and Boxaca, 1967; Borden and Murphy, 1971).

(b) RIP production is delayed in time relative to interferon production, as demonstrated in both animals and cell cultures (Borden and Murphy, 1971; Borden *et al.*, 1975). Thus, supernatant fluids collected 48 hr after infection of mouse L cells by Newcastle disease virus (NDV) contain a two to four fold higher ratio of RIP to interferon than supernatants collected at 14 hr.

(c) Judicious treatment of NDV-infected L cells with cycloheximide increases interferon production at the expense of RIP (Borden *et al.*, 1975).

(d) Supernatant fluids harvested from NDV-infected L cells, which have been pretreated with interferon, contain a significantly higher ratio of RIP to interferon than NDV-infected L cells which have not been treated with interferon (Chadha *et al.*, 1974). These results are clearly incompatible with the notion that refractoriness results primarily from a feedback inhibition of interferon production by interferon itself. The development of a refractory state apparently requires the formation of an additional factor (RIP) which may well be identical or similar to the repressor protein which accounts for the normal early shut-off of interferon production (and which will be mentioned in Section 15.4).

13. PRIMING: AN EFFECT OF INTERFERON TREATMENT ON INTERFERON PRODUCTION

The pretreatment of cells with interferon has been reported to have two opposing effects: (a) inhibition of interferon production; or (b) enhancement of interferon production. The latter phenomenon has been termed 'priming' (Isaacs and Burke, 1958) whereas the former has been referred to as 'blocking' (Golgher and Paucker, 1973). Enhancing effects of certain active or inactivated viruses may have also been due to production of small quantities of interferon (Burke and Isaacs, 1958; Ho and Breinig, 1962; Mahdy and Ho, 1964). Enhancement of interferon production by either virus or dsRNA by interferon pretreatment ($\leq$100 units/ml) was also reported by Friedman (1966), Levy *et al.* (1966), Soloviev *et al.* (1970), Rosztóczy and Mécs (1970), Stewart *et al.* (1971a, b, 1972a, b), Borden and Murphy (1971), Rousset (1974), Margolis *et al.* (1972), Giron *et al.* (1973), Tovell and Cantell (1971), Goore *et al.* (1973), Havell and Vilček (1972), Barmak and Vilček (1973), Levy-Koenig *et al.* (1970) and Rosztóczy (1971).* Higher doses of interferon have a 'blocking' effect (Vilček, 1962; Vilček and Rada, 1962; Borden and Murphy, 1971; Rousset, 1974; Youngner and Hallum, 1969; Stewart *et al.*, 1971a; Lockart, 1963; Friedman, 1966; Bausek and Merigan, 1970; Cantell and Paucker, 1963; Golgher and Paucker, 1973; Barmak and

*Levy-Koenig *et al.* (1970) also observed a priming effect with heterologous interferon; i.e. human interferon in mouse L cells.

Vilček, 1973). The time that the cells are exposed to the interferon is also of significance (Paucker and Boxaca, 1967). However, the effects of interferon pretreatment on subsequent interferon production is a complex phenomenon depending on both the cell type and inducer employed. Stewart *et al.* (1972b) found that interferon pretreatment had no effect on a certain line of mouse L cells that responded well to induction by $(I)_n \cdot (C)_n$, and Newcastle Disease Virus (NDV). This same line responded poorly to the MM strain of encephalomyocarditis virus, but the yield of interferon was dramatically enhanced if the cells were pretreated with interferon. In another study (Stewart *et al.*, 1971a), interferon pretreatment of a normally responding (to NDV or $(I)_n \cdot (C)_n$) cell line inhibited interferon production, but primary mouse kidney cells, although normally responding well to both $(I)_n \cdot (C)_n$ and NDV, were inhibited in their response to NDV but not to $(I)_n \cdot (C)_n$ when they were pretreated with interferon. A similar finding was reported by Margolis *et al.* (1972) who found that pretreatment of L cells with high or low doses of interferon did not inhibit interferon induction to $(I)_n \cdot (C)_n$ but did inhibit the interferon response to NDV.

The priming of interferon induction does appear to be a property of interferon itself rather than of an impurity in interferon preparations since interferon does not prime cells from heterologous species (Stewart *et al.*, 1971b), and the priming activity appears to copurify with the antiviral activity of interferon through a 10^6 increase in specific activity (Stewart *et al.*, 1973b).

Conflicting reports exist concerning the need of cellular protein synthesis for the priming activity to develop. On the one hand, the priming activity in chick embryo fibroblasts was inhibited by *p*-fluorophenylalanine (Friedman, 1966). On the other hand, Stewart *et al.* (1971b) found the priming effect in mouse L cells not to be inhibited by cycloheximide, *p*-fluorophenylalanine or puromycin, and Rosztóczy (1974) and Knight (1974b) found cycloheximide had no effect on priming even though, under similar conditions, the non-primed induction of interferon by $(I)_n \cdot (C)_n$ was inhibited. Again, the latter two studies were performed with L cells.

Pretreatment of cells with interferon invariably leads to an earlier interferon production (whether the final interferon yields are enhanced or reduced), and this interferon production becomes resistant to the effects of actinomycin D by 4–5 hr after induction, much sooner than production in normal cells (Levy *et al.*, 1966; Stewart *et al.*, 1971b). The kinetics of interferon production in primed cells, and the observation that cells exposed to interferon in the presence of protein synthesis inhibitors, still become fully primed (Stewart *et al.*, 1971b) has led to the suggestion that priming may be a non-antiviral function of interferon (Stewart *et al.*, 1971b; Rosztóczy, 1974). It is of interest, however, that both the priming activity of interferon and the antiviral state produced by interferon may be initiated from the cell surface since both states can be achieved with interferon bound to Sepharose (antiviral state: Ankel *et al.*, 1973; Knight, 1974a. priming: Knight, 1974b).

De Clercq *et al.* (1973b) found that treatment of L cells with pancreatic RNase brought about a relatively greater reduction of interferon production in interferon-primed cells than it did in unprimed cell cultures. However, priming did not enhance cell-binding of $(I)_n \cdot (C)_n$, did not alter the sensitivity of cell-associated $(I)_n \cdot (C)_n$ to RNase degradation, did not decrease the rate of degradation of cell-bound $(I)_n \cdot (C)_n$ nor did it increase the anti-nuclease potency of the cells. Thus, as with DEAE-dextran, interferon-priming is an experimentally useful, but poorly understood phenomenon.

14. BIOLOGICAL ACTIVITIES EXHIBITED BY LOW MOLECULAR WEIGHT INDUCERS OF INTERFERON AS COMPARED TO THOSE EXHIBITED BY DOUBLE-STRANDED RNAs AND POLYCARBOXYLATES

In addition to their *in vivo* antiviral activity which is largely accounted for by interferon production, low molecular weight inducers of interferon, such as tilorone

dihydrochloride, exert a wide variety of biological activities, most (but not all) of which correspond to the biological responses elicited by other interferon inducers (double-stranded RNAs and polycarboxylates). The different biological activities generated by tilorone dihydrochloride (as representative of the low molecular weight compounds), pyran copolymer (as representative of the polycarboxylates) and $(I)_n \cdot (C)_n$ (as representative of the double-stranded RNAs) are summarized in Table 20. While the induction of interferon and resistance to virus infection are dealt with in other sections of this review, we will now address the non-antiviral activities of tilorone and its counterparts of the polycarboxylate class and polynucleotide class of interferon inducers.

TABLE 20. *Review of Biologic Activities Exhibited by Low Molecular Weight Inducers of Interferon (Tilorone Dihydrochloride), Polycarboxylates (Pyran Copolymer) and Double-stranded RNAs* [$(I)_n \cdot (C)_n$]

Activity	Tilorone dihydrochloride	Pyran copolymer	$(I)_n \cdot (C)_n$
Interferon induction	+	+	+
Protection against viral infections	+	+	+
Direct antiviral activity	+	+	+
Inhibition of RNA-directed DNA polymerase activity of RNA tumor viruses	+	+	0
Inhibition of tumor growth	+	+	+
Adjuvant effect on humoral immunity	+	+	+
Adjuvant effect on cellular immunity	−	?	+
Stimulation of macrophage activity	0?	+	0?
Increase of body temperature	−	+	+
Lymphopenia, thrombocytopenia	+	+	+
Protection against bacterial infections	0, −	+	+
Protection against protozoal infections	?	+	+

+ denotes activity as indicated; 0 indicates absence of activity; and − denotes opposite activity. For references see text.

14.1. DIRECT INHIBITORY EFFECT ON VIRUS REPLICATION (NOT MEDIATED BY INTERFERON)

Tilorone dihydrochloride, pyran copolymer and $(I)_n \cdot (C)_n$ have all been shown to directly interfere with one or another step of the replication of some viruses in cultured cells. Tilorone inhibits the replication of herpes simplex virus in BSC-1 cells (Katz *et al.*, 1976a, b). The mechanism of this inhibition has not been resolved but may be related to a shut-off of viral DNA synthesis (due to binding of tilorone to the A–T rich regions of the viral DNA?). Pyran copolymer and its congeners, polyacrylic acid and polymethacrylic acid, prevent the replication of several viruses (vaccinia, vesicular stomatitis virus, . . .), in mouse, rabbit, rat or chick cells by virtue of an inhibition of virus adsorption to the host cell (De Somer *et al.*, 1968a; Merigan and Finkelstein, 1968). In well-defined conditions, $(I)_n \cdot (C)_n$ may directly interfere with viral RNA synthesis, e.g. in Detroit cells infected with poliovirus (Flikke *et al.*, 1970; Kjeldsberg and Flikke, 1971).

14.2. INHIBITION OF RNA-DIRECTED DNA POLYMERASE (REVERSE TRANSCRIPTASE) ACTIVITY OF RNA TUMOR VIRUSES

Tilorone dihydrochloride and some analogs of tilorone were found to be potent inhibitors of the RNA-directed DNA polymerases from oncogenic RNA viruses (Rauscher leukemia virus, Friend leukemia virus, Moloney sarcoma virus and avian myelobastosis virus) (Chandra *et al.*, 1972a, 1974; Schafer *et al.*, 1974; Green *et al.*, 1975). The most effective inhibitor was the fluoranthene derivative bis[3-diethylamino)-propyl]fluoranthene-3,9-dicarboxylate dihydrochloride (Chandra *et al.*, 1974; Green *et al.*, 1975). The latter compound is also a very efficient interferon inducer (Albrecht *et al.*, 1974b). Inhibition was primarily due to the planar ring structure of the

compound, and was also template-specific in the sense that inhibition was observed with some primer-templates, e.g. $(A)_n \cdot (dT)_{12-18}$, but not with others, e.g. $(C)_n \cdot (dG)_{12-18}$. It was suggested, therefore, that tilorone and its congeners inhibited DNA polymerase activity by binding to the template and not to the enzyme (Green *et al.*, 1975). The planar ring structure of tilorone permits intercalation between contiguous bases or base pairs in a helical duplex, and additional studies have indeed revealed that tilorone specifically interacts with A–T regions of double-stranded DNA (Chandra and Woltersdorf, 1976). As a result, the thermal stability and intrinsic viscosity of the DNA are increased, whereas its sedimentation rate is decreased (Chandra *et al.*, 1972b; Chandra *et al.*, 1972c; Chandra and Woltersdorf, 1974).

Unlike tilorone, $(I)_n \cdot (C)_n$ does not inhibit the DNA polymerase activity associated with RNA tumor viruses. In fact, $(I)_n$, which can be considered as a weak inhibitor of the RNA-directed DNA polymerase, loses its inhibitory effect upon complex formation with $(C)_n$ (De Clercq *et al.*, 1975a). On the contrary, inhibition of the RNA-directed DNA polymerase of avian myelobastosis virus was recorded for pyran copolymer (Papas *et al.*, 1974). Inhibition was not template-specific. Since pyran copolymer displayed kinetics of inhibition which was not competitive with the template, it was considered to inhibit DNA synthesis by binding to the DNA polymerase at a site other than the template site (Papas *et al.*, 1974).

14.3. Inhibition of Tumor Growth

Bis[3-(diethylamino)-propyl]-fluoranthene-3,9-dicarboxylate, the most potent reverse transcriptase inhibitor among the tilorone congeners, also emerged as the most efficient inhibitor of focus formation by Moloney sarcoma virus in normal mouse cells (Green *et al.*, 1975). In mice, tilorone, bis-[3-(diethylamino)-propyl]fluoranthene-3,9-dicarboxylate and some of their congeners were found to inhibit the development of Walker carcinosarcoma, reticulum cell sarcoma (Adamson, 1971), Friend leukemia and Ehrlich adenocarcinoma (Munson *et al.*, 1972). Similar antitumor activities have been repeatedly observed with pyran copolymer (as reviewed by De Clercq, 1973; see also Breslow *et al.*, 1973) and $(I)_n \cdot (C)_n$ (as reviewed by De Clercq, 1976).

14.4. Adjuvant Effect on Humoral Immunity

Tilorone enhances humoral antibody production in mice, e.g. in response to sheep red blood cells (Munson *et al.*, 1972; Diamantstein, 1973; Megel *et al.*, 1974). A similar immuno-adjuvant effect has been noted with pyran copolymer (Braun *et al.*, 1970) and a related polycarboxylate (chlorite-oxidized oxyamylose: Billiau *et al.*, 1971a) as well as with $(I)_n \cdot (C)_n$ (Turner *et al.*, 1970; Collavo *et al.*, 1972; Morahan *et al.*, 1972b). The adjuvant effect of tilorone, pyran copolymer and $(I)_n \cdot (C)_n$ on humoral antibody formation may be the result of B lymphocyte or macrophage stimulation. It is noteworthy that continued administration of tilorone to mice led to a significant increase in the relative number of B lymphocytes in the blood and spleen (Raychaudhuri and Megel, 1976).

14.5. Adjuvant Effect on Cellular Immunity

While increasing humoral antibody production, tilorone suppressed cell-mediated immunity, as evidenced by an inhibition of the tuberculin skin reaction and the direct passive Arthus reaction, and inhibition of adjuvant arthritis and experimental allergic encephalomyelitis in rats (Megel *et al.*, 1974; Megel *et al.*, 1975) and prolongation of the survival of allografts in mice (Mobraaten *et al.*, 1973). Tilorone is unique in combining a suppressive effect on cell-mediated immunity with an enhancing effect on humoral immunity. In this sense, it differs from established immunosuppressants (e.g. glucocorticoids), which are capable of suppressing both. By its suppressive effect on cellular immunity, tilorone deviates from $(I)_n \cdot (C)_n$ which, in addition to its adjuvant effect on humoral immunity, stimulates graft versus host activity and isograft re-

jections, two manifestations of cellular immunity (Cantor *et al.*, 1970; Turner *et al.*, 1970). The influence of pyran copolymer on the cell-mediated immune responses has not been assessed. The fact that pyran copolymer enhances the survival of some tumor allografts (Mohr *et al.*, 1976) could possibly be interpreted as evidence for an inhibitory effect of pyran copolymer on cellular immunity.

The mechanism by which tilorone exerts is suppressive effect on cell-mediated immunity is unknown. It may well be related to a selective depletion of T lymphocytes in the thymus-dependent areas of the spleen, lymph nodes and Peyer's patches (Levine *et al.*, 1974; Raychaudhuri and Megel, 1976).

14.6. Stimulation of Macrophage Activity

Some of its congeners (but not tilorone itself) stimulate reticuloendothelial macrophage cell activity, as measured by the intravascular clearance of colloidal carbon (Munson *et al.*, 1972). If effective at the macrophage level, tilorone cannot be considered as a very potent stimulus of macrophage activity, since it failed to potentiate the specific cytolytic effect of peritoneal macrophages on tumor cells (Schultz *et al.*, 1976). Under the same conditions, pyran copolymer greatly enhanced the specific tumoricidal activity of macrophages, as did some other substances (e.g. BCG). Pyran copolymer has profound effects on the reticuloendothelial system. Upon an initial suppression, it causes a dramatic increase of the phagocytic activity of the reticuloendothelial cells, as monitored by the disappearance of colloidal carbon from the blood stream and the uptake of sheep red blood cells by liver, spleen, lung and thymus (Munson *et al.*, 1970; Kapila *et al.*, 1971; Morahan *et al.*, 1972b). Although $(I)_n \cdot (C)_n$ may be suspected to alter the activity of the reticuloendothelial system, Morahan *et al.* (1972) did not observe a significant change in uptake of sheep red blood cells by liver, spleen, lung or thymus after administration of $(I)_n \cdot (C)_n$, most probably because the dosage of $(I)_n \cdot (C)_n$ was too low (1 mg/kg). With higher doses of $(I)_n \cdot (C)_n$ (~5 mg/kg) Chester *et al.* (1971) noted a biphasic effect on the carbon clearance: a decreased clearance rate if $(I)_n \cdot (C)_n$ was administered shortly (8–24 hr) before the injection of carbon; an increased clearance rate if $(I)_n \cdot (C)_n$ was administered 48–72 hr before carbon injection.

14.7. Increase of Body Temperature

In contrast with $(I)_n \cdot (C)_n$ and pyran copolymer which are both known to increase body temperature in rabbits and/or man (Merigan and Regelson, 1967; Lindsay *et al.*, 1969; Field *et al.*, 1971; Ts'o *et al.*, 1976), tilorone was found to depress body temperature in mice (Gláz and Tálas, 1975). The reasons for this discrepancy are not immediately clear. It should be pointed out, however, that for $(I)_n \cdot (C)_n$ and pyran copolymer, the rise in body temperature closely parallels serum interferon titers, whereas with tilorone, depression of body temperature occurs before interferon is being produced. In fact, by the time interferon starts to appear in the serum (6 hr after tilorone administration), body temperature has returned to normal (Gláz and Tálas, 1975).

14.8. Lymphopenia, Thrombocytopenia

Tilorone causes lymphopenia (Fibson *et al.*, 1976). Pyran copolymer causes thrombocytopenia (Merigan and Regelson, 1967) and $(I)_n \cdot (C)_n$ causes both lymphopenia and thrombocytopenia (Degré, 1973; Carter *et al.*, 1976). Following tilorone treatment, mice exhibit a dramatic reduction in peripheral lymphocyte number which reaches its nadir 24 hr after a single oral dose of tilorone; that is, at the time interferon output is maximal. The characteristic lymphopenia noted upon tilorone therapy is most likely due to a specific depletion of T lymphocytes (Gibson *et al.*, 1976).

14.9. Protection Against Bacterial Infections

Tilorone does not offer significant protection against lethal infections caused by extracellular bacteria such as *Staphylococcus aureus*, *Diplococcus pneumonia* and *Pasteurella tularensis* (Munson *et al.*, 1972; Giron *et al.*, 1972a). On the contrary, tilorone greatly increases the susceptibility of mice to infection with intracellular microorganisms such as *Listeria monocytogenes*, *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Salmonella enteritidis* (Collins, 1975; Gruenewald and Levine, 1976). The deleterious effect of tilorone on the host's resistance to intracellular microbial infections is probably related to the suppression of cell-mediated immunity by tilorone. To a major extent, the host's defense against intracellular parasites like *Listeria* indeed depends on cell-mediated immunity.

The other interferon inducers, pyran copolymer and $(I)_n \cdot (C)_n$, induce resistance to both extracellular and intracellular bacterial infections (Weinstein *et al.*, 1970; Remington and Merigan, 1970; Pindak, 1970; Giron *et al.*, 1972a).

14.10. Protection Against Protozoal Infections

Polycarboxylates, double-stranded RNAs and other interferon inducers are known to protect mice against protozoal infections such as *Plasmodium berghei* sporozoites (Jahiel *et al.*, 1968; Van Dijck *et al.*, 1970). Whether this protective activity is shared by tilorone has not been established.

15. MECHANISM OF INTERFERON INDUCTION

15.1. Is the Whole Antiviral Activity Exhibited by $(I)_n \cdot (C)_n$ Accounted for by Interferon Production?

To stimulate detectable amounts of interferon in the cell culture fluid significantly higher doses of dsRNA [$(I)_n \cdot (C)_n$] are required than for induction of cellular resistance to virus infection (Finkelstein *et al.*, 1968; Ho and Ke, 1970; De Clercq and Merigan, 1971a). There are, however, a number of observations which suggest that even at polymer concentrations which do not lead to the appearance of detectable amounts of interferon in the cell culture medium (Field *et al.* 1968; Vilček *et al.*, 1968; De Clercq and Merigan, 1969a) interferon is a necessary intermediate for the development of the antiviral state:

(a) Viruses which are susceptible to interferon are inhibited by $(I)_n \cdot (C)_n$, and viruses which are refractory to interferon also resist inhibition by $(I)_n \cdot (C)_n$ (Stewart *et al.*, 1969; Thacore and Youngner, 1973). For example, the replication of VSV (vesicular stomatitis virus) in RK-13, a continuous line of rabbit kidney cells, is suppressed by both interferon and $(I)_n \cdot (C)_n$. Neither interferon nor $(I)_n \cdot (C)_n$ affect the growth of vaccinia virus in RK-13 cells, although vaccinia virus is quite susceptible to interferon when assayed in other cells like mouse or chicken (Thacore and Youngner, 1973). Similarly, Hiller *et al.* (1973) found $(I)_n \cdot (C)_n$ and exogenous interferon equally effective in inhibiting 'early' viral protein synthesis in chick embryo fibroblasts infected with vaccinia virus.

(b) Cells which are unable to produce interferon, even though they may respond to interferon, do not become resistant to virus infection upon exposure to $(I)_n \cdot (C)_n$. For example, Vero cells (a continuous line of African green monkey cells), which respond to interferon but cannot produce it, do not become resistant to virus infection after $(I)_n \cdot (C)_n$ treatment, while LLC–MK_2 cells (another monkey kidney cell line), which both produce and respond to interferon, do become resistant after $(I)_n \cdot (C)_n$ treatment (Schafer and Lockart, 1970). McCoy cells that respond to interferon as do Vero cells, might constitute an exception to the rule established above, since McCoy cells have been reported to be protected by $(I)_n \cdot (C)_n$ in the absence of detectable interferon production (Giron *et al.*, 1972b). This does not imply, however, that in McCoy cells, $(I)_n \cdot (C)_n$ acts through a mechanism other than interferon induction. Careful inspection

of Giron's data reveals that McCoy cells, unlike L cells which produce relatively high interferon amounts in response to $(I)_n \cdot (C)_n$, are only partially protected by $(I)_n \cdot (C)_n$. In addition, this protective effect may be achieved by interferon that is stored intracellularly.

(c) If the inhibitory effect of $(I)_n \cdot (C)_n$ on virus multiplication is mediated by interferon production, one may expect T-21 cells (human fibroblasts trisomic for chromosome 21), which are more sensitive to the antiviral action of interferon than normal diploid (D-21) cells, also to be more sensitive to the antiviral action of $(I)_n \cdot (C)_n$. As shown by Tan *et al.* (1974b) and De Clercq *et al.* (1975b), $(I)_n \cdot (C)_n$ proved indeed more active in inhibiting virus replication in T-21 cells than in D-21 cells. The latter differences truly reflected differences in interferon sensitivity, not differences in interferon production, since $(I)_n \cdot (C)_n$ did not induce more interferon in T-21 cells than in D-21 cells (Tan *et al.*, 1974b).

(d) If $(I)_n \cdot (C)_n$ acts indirectly, that is through induction of interferon, it should be possible to affect differently the successive stages involved in the antiviral action of $(I)_n \cdot (C)_n$. Vilček and Varacalli (1971) clearly established that the development of cellular resistance to virus infection can indeed be blocked by actinomycin D at a time when interferon production in the same (rabbit kidney cell) cultures is no longer inhibited by the antimetabolite. Generally, $(I)_n \cdot (C)_n$ is added to the cell cultures several hours before virus inoculation. This procedure offers sufficient time for interferon-mediated resistance to develop. If, however, $(I)_n \cdot (C)_n$ is added at the time of virus inoculation, $(I)_n \cdot (C)_n$ might directly interfere with viral RNA synthesis, as has been demonstrated for poliovirus (Kjeldsberg and Flikke, 1971).

(e) Finally, anti-interferon antibody effectively neutralizes the antiviral activity of $(I)_n \cdot (C)_n$ (Vengris *et al.*, 1975). For the neutralization to be achieved, the anti-interferon serum has to be present in the cell culture medium from the time interferon is being released into the medium. Treatment with anti-interferon serum before exposure to $(I)_n \cdot (C)_n$ has no effect. These data indicate that the antiviral activity of $(I)_n \cdot (C)_n$ is interferon-mediated. They also led Vengris *et al.* (1975) to suggest that, for the development of the antiviral state, interferon has to interact with the external part of the cellular membrane, even in the cell in which it is produced.

15.2. KINETICS AND SPECIFICITY OF INTERACTION OF $(I)_n \cdot (C)_n$ WITH THE CELLS

The first step in the induction of interferon by a double-stranded RNA molecule such as $(I)_n \cdot (C)_n$ is the binding of $(I)_n \cdot (C)_n$ to the cell surface. This step is not temperature-dependent. It occurs equally well at 4 and 37°C (Bausek and Merigan, 1969). However, there does exist a difference in the disposition of the polymer at these two temperatures. When bound to the cells at 4°C, $(I)_n \cdot (C)_n$ can be destroyed by RNase treatment (Bausek and Merigan, 1969), neutralized by anti-$(I)_n \cdot (C)_n$ antibody (Vengris *et al.*, 1975) and washed off with high concentrations of salt or single-stranded polynucleotides (Johnston *et al.*, 1976). During the second stage, which occurs only at 37°C, the polynucleotide becomes inaccessible to the effects of RNase and anti-$(I)_n \cdot (C)_n$ antibodies.

This second, temperature-dependent, step occurs very rapidly after cell binding of $(I)_n \cdot (C)_n$ and leads to interferon production and cellular resistance to virus infection. Although full resistance to virus infection and maximum interferon synthesis is not attained until several hours after addition of $(I)_n \cdot (C)_n$ to the cells, the time of exposure of the double-stranded RNA to the cells may be very short: one minute exposure at either 4 or 37°C will suffice for antiviral resistance to develop (De Clercq *et al.*, 1971; Pitha *et al.*, 1972). Depending on the nature of the polymer and the polymer concentration, somewhat longer exposure times (up to 60 min) may be needed to obtain maximum interferon yields. Continued exposure of the cells to the polymer is unnecessary and can even be deleterious, since the polymer (or degraded material thereof) may interfere with normal cell metabolism (e.g. DNA, RNA or protein synthesis) (De Clercq *et al.*, 1976a).

Cells take up excessive amounts of $(I)_n \cdot (C)_m$. The bulk of the cell-associated $(I)_n \cdot (C)_n$ persists at the cell surface. Very little $(I)_n \cdot (C)_n$ is taken up into the cell upon a 1 hr exposure period (Bausek and Merigan, 1969). For some cell cultures (viz. those particularly sensitive to the antiviral action of dsRNAs), intact $(I)_n \cdot (C)_n$ may be recovered in the homogenates of cells after they have been exposed to the dsRNA (De Clercq and Stewart, 1974). Only a small fraction of the cell-associated $(I)_n \cdot (C)_n$ (whether located at the cell surface or penetrated into the cell) appears to be essential for interferon production, as suggested by the following observations:

(a) With a variety of polyribo- and polydeoxyribonucleotides, including $(I)_n \cdot (C)_n$, $(dI)_n \cdot (dC)_n$, $(A)_n \cdot (U)_n$, $(dA)_n \cdot (dT)_n$, (I–C)$_n$, (dI–dC)$_n$, (A–U)$_n$, and (dA–dT)$_n$, all assayed in the same cells (human skin fibroblasts), no direct correlation could be established between the amounts of polynucleotide bound to the cells and the antiviral activity exhibited by these polynucleotides (De Clercq *et al.*, 1972b).

(b) No significant differences were noted in the amounts of $(I)_n \cdot (C)_n$ associated with a variety of cells, including primary rabbit kidney, human skin fibroblast, mouse embryo fibroblast, mouse L, rabbit kidney RK 13, HeLa, BSC-1 and Vero cells, although the antiviral activity exhibited by $(I)_n \cdot (C)_n$ in these cells varied considerably from one cell system to another (De Clercq and De Somer, 1973b). In these studies (De Clercq *et al.*, 1972b; De Clercq and De Somer, 1973b), cell-binding of the polymers was studied over a 1 hr time period, since a 1 hr exposure period largely suffices to initiate maximum interferon production. In similar studies, Field *et al.* (1972b) found no preferential uptake of $(I)_n \cdot (C)_n$ by primary rabbit kidney cells as compared to that of the single homopolymers $(I)_n$ and $(C)_n$. Also, Colby and Chamberlin (1969) failed to observe differences in the kinetics of cellular uptake between an active interferon inducer, $(I)_n \cdot (C)_n$, erroneously referred to as (I–C)$_n$, and an inactive inducer, $(I)_n \cdot (dC)_n$, and Schafer and Lockart (1970) failed to demonstrate differences in the rate of binding of $(I)_n \cdot (C)_n$ to cells which respond to the antiviral action of $(I)_n \cdot (C)_n$ (LLC–MK 2 cells) and cells which do not respond (Vero cells). In the latter two studies, however, the kinetics of cell-binding were explored over an extended period of time (24 hr). Therefore these studies do not directly bear on the interferon induction process which is fully triggered within 1 hr.

(c) Concanavalin A, a plant lectin which causes specific alterations of the cell membrane, was found to inhibit interferon induction by $(I)_n \cdot (C)_n$ in both mouse and human fibroblasts, while not affecting the binding of the polymer to the cells (Harper and Pitha, 1973). Similarly, treatment of the cells with neuraminidase or phospholipase C at concentrations which completely suppressed interferon induction by $(I)_n \cdot (C)_n$, did not affect binding of the inducer to the cells (Pitha *et al.*, 1974). These results indicate that the majority of the $(I)_n \cdot (C)_n$ molecules bound to the cell are bound at nonspecific sites which are not involved in interferon induction.

Another observation which may be more specifically related to the interferon induction process is that when $(I)_n$ and $(C)_n$ are added sequentially to the cells, the double-stranded complex $(I)_n \cdot (C)_n$ is formed at the cell surface (De Clercq and De Somer, 1972a); the double-helical complex formed *in situ* at the cell membrane is more firmly bound to the cell than when added exogenously (De Clercq *et al.*, 1973a; Johnston *et al.*, 1976) and, concomitantly, the complex formed *in situ* is more effective in eliciting an interferon response than the complex added exogenously (De Clercq *et al.*, 1973a). It remains to be established, however, whether this increased interferon response is directly related to a better interaction of the polymer with the (putative) receptor site for interferon induction.

15.3. Major Events in the Interferon Induction Process

Most pertinent information regarding the mechanism of interferon induction arises from the use of $(I)_n \cdot (C)_n$ as the model inducer. One hypothetical mechanism of interferon induction is presented schematically in Fig. 17 and can be divided into four parts. The first step could be the interaction of the inducer with a specific cellular receptor site (possibly located at the cell's outer membrane). This interaction would

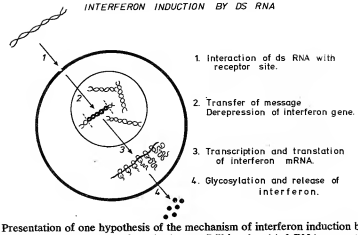


FIG. 17. Presentation of one hypothesis of the mechanism of interferon induction by dsRNA. Other, equally tenable hypotheses include the possibilities that (a) dsRNA generates a signal that leads to derepression of interferon mRNA or the processing of pre-interferon mRNA; or (b) that dsRNA itself binds to a repressor of interferon mRNA, thus freeing it for translation.

generate a message which would be transmitted into the interior of the cell, leading to derepression of the interferon gene, transcription to give the interferon mRNA, translation of the interferon mRNA into the interferon protein, and, eventually, glycosylation and secretion of the interferon molecule. There is circumstantial evidence for all these steps, except the second one (transfer of the message from the receptor site to the cellular genome). The timing and sites of the successive events in the interferon induction process are depicted in Table 21. There are a number of equally valid alternative hypothesis to the one presented in Fig. 17. For instance, Ng and Vilček (1972) suggested that dsRNA (or $(I)_n \cdot (C)_n$) binds to the interferon mRNA repressor, thus freeing the mRNA for translation.

Since antibodies to dsRNA recognize many of the structural features that are responsible for the interferon inducing capacity of dsRNAs, it has been postulated that the cellular receptor site for polynucleotide inducers of interferon may be protein in nature (Johnston *et al.*, 1975; see also Colby and Chamberlin, 1969). Is this (putative) receptor site located at the outside of the cell or inside the cell? Studies with $(I)_n \cdot (C)_n$ attached to cells [mouse L cells (De Clercq and De Somer, 1972b), rabbit red blood cells (De Clercq and De Somer, 1974b)] or covalently coupled to insoluble supports [Sepharose (Wagner *et al.*, 1971; Taylor-Papadimitriou and Kallos, 1973; Bachner *et al.*, 1975), cellulose, sephadex (Pitha and Pitha, 1973) or cellophane (Hutchinson and Merigan, 1975)] suggest, but do not prove, that the receptor site for interferon induction by $(I)_n \cdot (C)_n$ may be situated at the cell surface. Leakage of polynucleotide from the matrix during its incubation with the cells has been observed with all insolubilized $(I)_n \cdot (C)_n$ reparations employed so far. This leakage precludes an unequivocal answer to the question as to whether the polynucleotide must penetrate into the cell in order to trigger the induction of interferon.

TABLE 21. *Sequence of Major Events During Interferon Induction*[a] *by* $(I)_n \cdot (C)_n$

Approximate time (hr)	Event	Site
0–0.5	Triggering	Receptor on Plasma membrane(?)
0.5–2	Transcription of interferon mRNA	Nucleus
1–3.5	Translation of interferon mRNA	Rough ER (or free polysomes?)
1–3.5	Glycosylation of interferon	Rough or smooth ER(?) or Golgi apparatus(?)
1.5–4.0	Release of interferon	Plasma membrane (via secretory vesicles?)
2–?	Transcription, translation and build-up of repressor	Nucleus and cytoplasm
3–?	Inactivation of interferon mRNA by repressor	Cytoplasm

[a] In human or rabbit cell cultures (according to Vilček *et al.*, 1975).

According to Fig. 17, after the receptor has been triggered by the interferon inducer, a signal is transmitted to the cellular genome and, as a result, the interferon gene is switched on. Direct evidence for this stage of the interferon induction process is lacking. The fact is that within 2 hr after exposure of the cells to $(I)_n \cdot (C)_n$, most of the interferon mRNA has been transcribed (Vilček and Havell, 1973). Indirect evidence for the existence of interferon mRNA comes from studies with metabolic inhibitors (actinomycin D, cycloheximide) (Vilček, 1970a). Direct proof for the existence of interferon mRNA was provided by De Maeyer-Guignard *et al.* (1972) who demonstrated that mRNA extracted from mouse cells, which had been induced to produce interferon either by Newcastle disease virus or by $(I)_n \cdot (C)_n$, could be translated into mouse interferon in heterologous (chick) cells. In mouse L cells induced by Newcastle disease virus, two kinds of interferon mRNAs are induced: a single-stranded mRNA containing $(A)_n$ and a 'double-stranded' mRNA lacking $(A)_n$; in L cells induced by $(I)_n \cdot (C)_n$, only the double-stranded type is formed (Montagnier *et al.*, 1974). The translation of mouse interferon mRNA isolated from $(I)_n \cdot (C)_n$-induced L cells in heterologous (Vero) cells has also been reported by Kronenberg and Friedmann (1975). These authors found that at appropriate RNA concentrations the yield of interferon was proportional to the concentration of RNA adsorbed to the recipient cells. Using the translation assay in chick cells, Reynolds and Pitha (1974) were able to demonstrate the presence of a $(A)_n$-rich interferon mRNA in human fibroblasts induced by $(I)_n \cdot (C)_n$. More recently, the translation of both mouse and human interferon mRNA was achieved in *Xenopus laevis* oöcytes as well as in cell-free ribosomal systems prepared from mouse Ehrlich ascites cells, Krebs II ascites cells, rabbit reticulocytes and wheat germ (Reynolds *et al.*, 1975; Pestka *et al.*, 1975; Thang *et al.*, 1975).

No interferon mRNA has been found in non-induced cells. It spresence in cells induced by viruses and $(I)_n \cdot (C)_n$ clearly indicates that the induction of interferon by viruses, double-stranded RNAs and possibly all other inducers, represents *de novo* synthesis. On this basis, the distinction between the so called endotoxin-type of interferon production (release of preformed interferon) to which the induction of interferon by $(I)_n \cdot (C)_n$ would belong (Youngner and Hallum, 1968)—and the virus-type of interferon production (newly synthesized interferon) (Youngner *et al.*, 1965) may no longer be tenable.

Proteins made for export from the cell are, as a rule, synthesized on membrane-bound polysomes (rough endoplasmic reticulum). Much of the intracellular interferon is indeed membrane-associated (Ng *et al.*, 1972); however, the actual site of interferon synthesis has not been elucidated. Since 2-deoxy-D-glucose and D-glucosamine, two inhibitors of glycosylation, inhibit interferon production, it is reasonable to assume that interferon is glycosylated before its release (Havell *et al.*, 1975b). After interferon has attained a sufficiently high concentration within the cell, it is released into the extracellular environment (Tan *et al.*, 1971a). The release of interferon from the cells is a temperature- and energy- requiring process. This process is not affected by protein synthesis inhibitors but is inhibited by SH-blockers such as *p*-hydroxymercuribenzoate (Tan *et al.*, 1972). Although the cellular organelles which participate in the release of interferon have not been established, it is noteworthy that interferon secretion is specifically suppressed by vinblastine, an alkaloid with high affinity for microtubules (Havell and Vilček, 1975).

15.4. Post-transcriptional Control of Interferon Synthesis

In human and rabbit fibroblast cultures, interferon production peaks approximately 3–4 hr after exposure of the cells to $(I)_n \cdot (C)_n$. By 6–8 hr, the production of interferon has ceased (Vilček *et al.*, 1975). However, when inhibitors of RNA and/or protein synthesis are added to the cell cultures at the appropriate times and dosages, interferon production does not stop, and the interferon yields are increased up to 1000-fold. The paradoxical enhancement of interferon production by the judicious use

of inhibitors of RNA and/or protein synthesis has been referred to as 'superinduction' (Fig. 18).

When added prior to or simultaneously with the interferon inducer at sufficiently high doses, metabolic inhibitors, such as cycloheximide, actinomycin D, pyromycin, *p*-fluorophenylalanine and 5,6-dichloro-1-(β-D-ribofuranosyl)-benzimidazole, suppress interferon production (Field *et al.*, 1968; Finkelstein *et al.*, 1968; Vilček *et al.*, 1969; Tan *et al.*, 1970; Ho and Ke, 1970; Myers and Friedman, 1971; Sehgal *et al.*, 1976c). However, when added some time after the interferon inducer, low to moderate doses of the metabolic inhibitor may actually enhance ('superinduce') the interferon response. Nearly 'classical' superinducers are cycloheximide and actinomycin D. They have been reported to accentuate interferon production in both rabbit kidney and human diploid fibroblasts (Vilček *et al.*, 1969; Tan *et al.*, 1970, 1971b; Vilček and Ng, 1971; Myers and Friedman, 1971; Ho *et al.*, 1972; Havell and Vilček, 1972; Vilček and Havell, 1973; Sehgal *et al.*, 1976a; Sehgal and Tamm, 1976). Other compounds which have been found to enhance interferon formation include toyocamycin (Ng and Vilček, 1973), 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (Sehgal *et al.*, 1975a, 1976a, b; Sehgal and Tamm, 1976) and two lysosomotropic drugs, neutral red and chloroquine (Sehgal *et al.*, 1975b). The 'superinducing' effect of all these agents appeared to be closely linked to the inhibition of RNA and/or protein synthesis. $(I)_n \cdot (C)_n$-induced cells, treated continuously with 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole, continued to produce interferon for 4 days (Sehgal *et al.*, 1976a).

Removal of 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole at any time during this period led to prompt shut-off of interferon production and an equally prompt recovery of cellular RNA synthesis (Sehgal *et al.*, 1976b). The lysosomotropic drugs, neutral red and chloroquine, are known to inhibit intralysosomal degradation of protein; however, their 'superinducing' effect was attributed to an inhibition of protein (and RNA) synthesis and not to an inhibition of intralysosomal proteolysis (Sehgal *et al.*, 1975b).

How could the superinduction of interferon production by inhibitors of protein and RNA synthesis be explained? Vilček and Ng (1971) postulated that inhibitors of protein (and RNA) synthesis suppress the formation of a cellular regulatory protein (repressor) which would otherwise interfere with the translation of the interferon mRNA. Thus, the (hypothetical) repressor molecule would control interferon synthesis at a post-transcriptional level. One could hardly visualize an action at the transcriptional level. If the repressor inhibited the transcription of interferon mRNA,

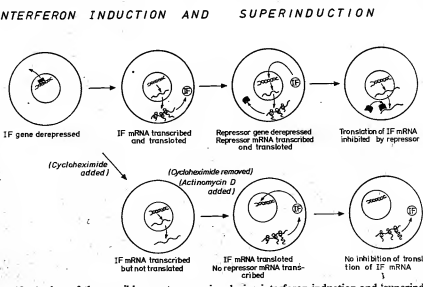


FIG. 18. A view of the possible events occurring during interferon induction and 'superinduction'. See text for details.

one would not expect actinomycin D to enhance interferon production, since actinomycin D is assumed to disrupt all DNA-dependent RNA synthesis, including repressor mRNA and interferon mRNA. How then would the repressor system interact with the translation of the interferon molecule? With the information presently available, the mode of action of the repressor can only be speculated upon. The shut-off of interferon production cannot be attributed to a simple competition by newly synthesized mRNA (Sehgal and Tamm, 1976). The results obtained with neutral red and chloroquine (Sehgal *et al.*, 1975b) indicate that interferon is not prematurely degraded, at least not intralysosomally. There are, however, several other possibilities to be considered. For example, the (putative) repressor system may control the processing of the primary interferon mRNA transcripts to active cytoplasmic mRNA. It may facilitate the degradation of the interferon mRNA in the nucleus and/or cytoplasm or hamper its transport from nucleus to cytoplasm. In the cytoplasm, the repressor may fix the interferon mRNA in an inactive form, prevent the formation of a functional ribosome-mRNA initiation complex or slow down the elongation rate. One might even envisage the repressor to operate at the post-translational level. Such a possibility should be entertained if it developed that the interferon molecule requires some post-translational modification (e.g. proteolytic cleavage) in order to be biologically active.

To delineate the mechanism by which the cell regulates interferon synthesis, it may seem useful to have a (cell-free protein synthesis) system at hand, that permits the direct quantitation of interferon mRNA. As reported by Reynolds and Pitha (1974), the increase in the amounts of interferon produced in 'superinduced' cells is not paralleled by a commensurate increase in the amounts of interferon mRNA. Such data provide rather compelling evidence that interferon production is regulated at the translational (or post-translational) level.

16. CONCLUSION

A number of substances which are effective inducers of interferon both *in vitro* and *in vivo* have been described herein; nonetheless, no clinical fulfilment of the promise of interferon inducers seems imminent. Two major obstacles continue to plague all efforts at interferon induction in humans: inducers exhibiting dramatic interferon-inducing activity in animal models fail to give encouraging levels of interferon in man; the toxic manifestations of inducers contraindicate inducer dosages or regimes that might possibly show an enhanced interferon response.

Double-stranded polyribonucleotides and (some) RNA viruses are by far the most potent interferon inducers, surpassing in activity all other interferon inducers such as low molecular weight inducers, polycarboxylates, endotoxins and stimulators of B or T lymphocytes. As inducers of interferon, synthetic polynucleotides are clearly advantageous to viruses: they do not pose biohazard problems and their chemical structure is amenable to further modifications.

For therapeutic purposes, synthetic polynucleotides could be employed either directly or indirectly. The direct approach implies that interferon is induced endogenously, upon direct administration of the inducer to man. To hold promise as an endogenous interferon inducer, the polynucleotide should be devoid of its undesirable toxic side effects. Alternatively, interferon inducers may demonstrate only indirect utility, that is, to induce interferon in cell culture. This interferon could then be administered exogenously to man. When used solely to stimulate interferon production *in vitro*, the polynucleotide need not be freed from its toxic side effects.

The induction of interferon by polynucleotides should not be regarded as an isolated phenomenon. Insofar as the structural features which govern the interferon response also to apply to other biological properties (e.g. mitogenic activity, inhibition of protein synthesis, etc.), interferon induction studies with polynucleotides bear directly on the general biological role of polynucleotides in the cell, e.g. with regard to the regulation of gene expression.

For the development of a 'superior' polynucleotide inducer of interferon, several different strategies could be followed. First, full advantage may be taken of the structural requirements that underlie the interferon-inducing activity of polynucleotides; i.e. construction of polynucleotide duplexes with a perfectly matched base-paired structure, maximal molecular weight, supreme resistance to enzymatic hydrolysis, etc. (see Section 10). The $(I)_n \cdot (C)_n$-poly(L-lysine)carboxymethylcellulose complex could be considered one example of such an approach. This complex resists degradation by primate serum, induces interferon in primates (Levy *et al.*, 1975), protects monkeys against simian hemorrhagic fever (Levy *et al.*, 1976) and modifies the course of chronic hepatitis B infection in chimpanzees (Purcell *et al.*, 1976). Another example is $(I)_n \cdot (s^2C)_n$ (Reuss *et al.*, 1976). The introduction of a single chemical modification into $(I)_n(C)_n$ produces a potent interferon-inducing complex with markedly enhanced nuclease resistance. A second approach for designing nucleic acids of greater interferon-inducing efficacy rests upon the basic premise that the kinetics of the interferon response and the other, undesirable, responses may not be identical. On this basis, polynucleotides could be developed which: (1) would persist long enough in biological fluids so as to permit the induction of interferon but (2) would not persist so long as to elicit any toxic side-effects. The mismatched analogs, $(I)_n \cdot (C_x U)_n$ and $(I)_n \cdot (C_x G)_n$, at least partially fulfill the premises of this latter approach, since these mismatched analogs, while comparable in their interferon-inducing properties to $(I)_n \cdot (C)_n$, showed less pronounced secondary effects (Ts'o *et al.*, 1976; Carter *et al.*, 1976). To the extent, however, that prophylaxis or therapy with interferon-inducers requires the maintenance of a high serum interferon level, these complexes may not be ideal since they would have to be administered repeatedly, thus defeating the purpose of their brief serum half-life. A third possible approach is entirely speculative in nature and is based on one possible biochemical restatement of the longstanding question: are interferon-induction and toxicity invariably linked? On the one hand, does interaction of an interferon inducer with its receptor trigger an irrevocable cascade of events, some of which are beneficial (e.g. interferon induction) and some of which are deleterious (e.g. protein synthesis inhibition)? On the other hand, might induction of interferon be triggered by the interaction of the inducer with one distinct receptor, and might the toxic manifestation(s) be triggered by interaction of the inducer with another different receptor? If the latter possibility obtained, then separation of interferon inducing ability from toxicity should be possible by chemical modifications that could selectively affect the inducer's affinity for one particular receptor.

There is no reason to be pessimistic regarding the eventual application of interferon-inducers and polynucleotides in medicine. It is remarkable that we have come this far when the magnitude of the problem is appreciated: a catalog of the variety of biological responses of interferon inducers and synthetic polynucleotides has not yet been completed; moreover, we know very little of the physical, chemical, and biochemical basis of just one of these responses, interferon induction. At the least, we can hope that the next few years would bring substantive increases in our knowledge of the myriad biological responses of interferon inducers, the mechanistic relation of one class of inducers to another, and some idea how these phenomena relate to the basic workings of the cell.

REFERENCES

ADAMSON, R. H. (1971) Antitumor activity of tilorone hydrochloride against some rodent tumors: preliminary report. *J. Nat. Cancer Inst.* **46**: 413–434.

AKSENOV, O. A., TIMKOWSKY, A. L., AGEEVA, O. N., KOGAN, E. M., BRESLER, S. E., SMORODINSTEV, A. A. and TIKHOMIROVA-SIDOROVA, N. S. (1973) Interferon-inducing and antiviral activities of the double-stranded polyriboguanylic acid-polyribocytidylic acid complex. *Vop. Virus* **18**: 345–349.

ALBERT, A. (1973) *Selective Toxicity*, pp. 130–172, 5th edn. Chapman and Hall, London.

ALBRECHT, W. L., FLEMING, R. W., HORGAN, S. W., DECK, B. A., HOFFMAN, J. W. and MAYER, G. D. (1974b) Bis-basic-substituted polycyclic aromatic compounds. A new class of antiviral agents. 6. Bis-basic substituted fluoranthenes. *J. Med. Chem.* **17**: 1150–1156.

ALBRECHT, W. L., FLEMING, R. W., HORGAN, S. W., KIHM, J. C. and MAYER, G. D. (1974a) Bis-basic-substituted polycyclic aromatic compounds. A new class of antiviral agents. 3. 2,7-Bis(aminoacyl) fluorenes and -fluorenones. *J. Med. Chem.* **17**: 886–889.

ANDREWS, E. R., FLEMING, R. W., GRISAR, J. M., KIHM, J. C., WENSTRUP, D. L. and MAYER, G. D. (1974) Bis-basic-substituted polycyclic aromatic compounds. A new class of antiviral agents. 2. Tilorone and related bis-basic ethers of fluorenone, fluorenol and fluorene. *J. Med. Chem.* **17**: 882–886.

ANKEL, H., CHANY, C., GALLIOT, B., CHEVALIER, M. J. and ROBERT, M. (1973) Antiviral effect of interferon covalently bound to Sepharose. *Proc. natn. Acad. Sci. USA* **70**: 2360–2363.

ARNOTT, S. (1970) The geometry of nucleic acids. *Prog. Biophys. molec. Biol.* **21**: 267–319.

ARNOTT, S. and BOND, P. J. (1973a) Structures for poly(U)·poly(A)·poly(U) triple stranded polynucleotides. *Nature New Biol.* **244**: 99–101.

ARNOTT, S. and BOND, P. J. (1973b) Triple-stranded polynucleotide helix containing only purine bases. *Science* **181**: 68–69.

ARNOTT, S., CHANDRASEKARAN, R., HUKINS, D. W. L., SMITH, P. J. C. and WATTS, L. (1974b) Structural details of a double-helix observed for DNA's containing alternating purine and pyrimidine sequences. *J. molec. Biol.* **88**: 523–533.

ARNOTT, S., CHANDRASEKAREN, R., and MARTTILA, C., (1974a) Structure for polyinosinic and polyguanylic acids. *Biochem. J.* **141**: 537–543.

ARNOTT, S., CHANDRASEKAREN, R. and SELSING, E. (1975) The variety of polynucleotide helices. In: *Structure and Conformation of Nucleic Acids and Protein–Nucleic Acid Interactions*, pp. 577–596, SUNDARALINGAM, M. and RAO, S. T., (eds), University Park Press, Baltimore.

ARNOTT, S., HUKINS, D. W. L., DOVER, S. D., FULLER, W. and HODGSON, A. R. (1973) Structures of synthetic polynucleotides in the A-RNA and A'-RNA conformations: X-ray diffraction analysis of the molecular conformations of polyadenylic acid·polyuridylic acid and polyinosinic acid·polycytidylic acid. *J. molec. Biol.* **81**: 107–122.

ATHERTON, K. T. and BURKE, D. C. (1975) Interferon induction by viruses and polynucleotides: a differential effect of camptothecin. *J. gen. Virol.* **29**: 297–304.

ATKINS, G. J., JOHNSTON, M. D., WESTMACOTT, L. M. and BURKE, D. C. (1974) Induction of interferon in chick cells by temperature-sensitive mutants of sindbis virus. *J. gen. Virol.* **25**: 381–390.

ATKINS, G. J. and LANCASHIRE, C. L. (1976) The induction of interferon by temperature-sensitive mutants of sindbis virus: its relationship to double-stranded RNA synthesis and cytopathic effect. *J. Gen. Virol.* **30**: 157–165.

AZUMA, M. (1973) Participation of cytophilic antibody in enhancement of interferon production in macrophages by underneutralized NDV. *Arch. Ges. Virusforsch.* **41**: 11–19.

AZUMA, M., YAMADA, M. and NISHIOKA, R. (1970a) Relationship between immunity and interferon production in macrophages, II. Molecular weights of interferon produced in macrophage cultures. *Jap. J. Microbiol.* **14**: 319–323.

AZUMA, M., YAMADA, M. and NISHIOKA, R. (1970b) Relationship between immunity and interferon production in macrophages, III. Enhancement of interferon production by antiserum. *Jap. J. Microbiol.* **14**: 325–331.

BABIUK, L. A. and ROUSE, B. T. (1976) Immune interferon production by lymphoid cells: role in the inhibition of herpes-viruses. *Infect. Immunity* **13**: 1567–1578.

BACHNER, L., DE CLERCQ, E. and TRANG, M. N. (1975) Sepharose-bound poly(I)·poly(C): interaction with cells and interferon production. *Biochem. biophys. Res. Commun.* **63**: 476–483.

BADGER, A., COOPERBAND, S. R. and GREEN, J. A. (1972) Effect of polyriboinosinic–polyribocytidylic acid on the metabolism and viability of cells *in vitro*. *J. natn. Cancer Inst.* **49**: 613–621.

BAKAY, M. and BURKE, D. C. (1972) The production of interferon in chick cells infected with DNA viruses: a search for double-standard RNA. *J. gen. Virol.* **16**: 399–403.

BANKS, G. T., BUCK, K. W., CHAIN, E. B., DARBYSHIRE, J. E. and HIMMELWEIT, F. (1969) *Penicillium cyaneo-fulvum* virus and interferon stimulation. *Nature* **223**: 155–158.

BANKS, G. T., BUCK, K. W., CHAIN, E. B., DARBYSHIRE, J. E., HIMMELWEIT, F., RATTI, G., SHARPE, T. J. and PLANTEROSE, D. N. (1970) Antiviral activity of double-stranded RNA from a virus isolated from *Aspergillus foetidus*. *Nature* **227**: 505–507.

BANKS, G. T., BUCK, K. W., CHAIN, E. B., HIMMELWEIT, F., MARKS, J. E., TYLER, J. M., HOLLINGS, M., LAST, F. T. and STONE, O. M. (1968) Viruses in fungi and interferon stimulation. *Nature* **218**: 543–545.

BARMAK, S. L. and VILCEK, J. (1973) Altered cellular responses to interferon induction by poly(I)·poly(C): priming and hyporesponsiveness in cells treated with interferon preparations. *Arch. Ges. Virusforsch* **43**: 272–283.

BARNARD, E. A. (1969) Ribonucleases. *A. Rev. Biochem.* **38**: 677–732.

BARON, S., BOGOMOLOVA, N. N., BILLIAU, A., LEVY, H. B., BUCKLER, C. E., STERN, R. and NAYLOR, R. (1969) Induction of interferon by preparations of synthetic single-stranded RNA. *Proc. natn. Acad. Sci. USA* **64**: 67–74.

BARON, S., DE BUY, H., BUCKLER, C. E., JOHNSON, M. L., PARK, H., BILLIAU, A., SARMA, P. and HUEBNER, R. J. (1970) Induction of interferon and viral resistance in animals by polynucleotides. *Ann. N.Y. Acad. Sci.* **173**: 568–581.

BARTFELD, H. and VILCEK, J. (1975) Immunologically specific production of interferon in cultures of rabbit blood lymphocytes: association with *in vitro* tests for cell-mediated immunity. *Infect. Immunity* **12**: 1112–1115.

BASES, R. and KAPLAN, B. H. (1973) Double-stranded RNA from HeLa cell nuclei inhibits initiation of protein synthesis. *Biochim. biophys. Acta* **312**: 574–580.

BAUSEK, G. H. and MERIGAN, T. C. (1969) Cell interaction with a synthetic polynucleotide and interferon production *in vitro*. *Virology* **39**: 491–498.

BAUSEK, G. H. and MERIGAN, T. C. (1970) Two mechanisms of cell resistance to repeated stimulation of interferon. *Proc. Soc. exp. Biol. Med.* **134**: 672–676.

BEVAN, E. A., HERRING, A. J. and MITCHELL, D. J. (1973) Preliminary characterization of two species of dsRNA in yeast and their relation to the 'killer' character. *Nature* **245**: 81–86.

BILLIAU, A. (1970) The refractory state after induction of interferon with double-stranded RNA. *J. gen. Virol.* **7**: 225–232.

BILLIAU, A., BUCKLER, C. E., DIANZANI, F., UHLENDORF, C. and BARON S. (1969) Induction of the interferon mechanism by single-stranded RNA: potentiation by polybasic substances. *Proc. Soc. exp. Biol. Med.* **132**: 790–796.

BILLIAU, A., DESMYTER, J. and DE SOMER, P. (1970a) Antiviral activity of chlorite-oxidized oxyamylose, a polyacetal carboxylic acid. *J. Virol* **5**: 321–328.

BILLIAU, A., EYSSEN, H., DAVID, G. and DE SOMER, P. (1971a) Adjuvant activity of polyacetal carboxylate for antibody induction in mice and guinea pigs. *Proc. Soc. exp. Biol. Med.* **137**: 626–630.

BILLIAU, A., MUYEMBE, J. J. and DE SOMER, P. (1971b) Effect of chlorite-oxidized oxyamylose on influenza virus infection in mice. *Appl. Microbiol.* **21**: 580–584.

BILLIAU, A., MUYEMBE, J. J. and DE SOMER, P. (1971c) Mechanism of antiviral activity *in vivo* of polycarboxylates which induce interferon production. *Nature New Biol.* **232**: 183–186.

BILLIAU, A., MUYEMBE, J. J. and DE SOMER, P. (1972a) Interferon-inducing polycarboxylates: mechanism of protection against vaccinia virus infection in mice. *Infect. Immunity* **5**: 854–857.

BILLIAU, A., SCHONNE, E., EYCKMANS, L. and DE SOMER P. (1970b) Interferon induction and resistance to virus infection in mice infected with *Brucella abortus*. *Infect. Immunity* **2**: 698–704.

BILLIAU, A., VAN DER BERGHE, H. and DE SOMER, P. (1972b) Increased interferon release and morphological alteration in human cells by repeated exposure to double-stranded RNA. *J. gen. Virol* **14**: 25–31.

BLACK, D. R., ECKSTEIN, F., DE CLERCQ, E. and MERIGAN, T. C. (1973) Studies on the toxicity and antiviral activity of various polynucleotides. *Antimicrob Ag. Chemother.* **3**: 198–206.

BLACK, D. R., ECKSTEIN, F., HOBBS, J. B., STERNBACH, H. and MERIGAN, T. C. (1972) The antiviral activity of certain thiophosphate and 2'-chloro substituted homopolymer duplexes. *Virology* **48**: 537–545.

BLAKE, R. D., MASSOULIÉ, J. and FRESCO, J. R. (1967) Polynucleotides VIII. A spectral approach to the equilibria between poly(A) and poly(U) and their complexes. *J. molec. Biol.* **30**: 291–308.

BLOMGREN, H., STRANDER, H. and CANTELL, K. (1974) Effect of human leukocyte interferon on the response of lymphocytes to mitogenic stimuli *in vitro*. *Scand. J. Immunol.* **3**: 697–705.

BLOOMFIELD, V. A., CROTHERS, D. M. and TINOCO, I. M. (1974) *Physical Chemistry of Nucleic Acids*. Harper and Row, New York.

BOBST, A. M., TORRENCE, P. F., KOUIDOU, S. and WITKOP, B. (1976) Dependence of interferon induction on nucleic acid conformation. *Proc. natn. Acad. Sci. USA* **73**: 3788–3792.

BORDEN, E. C. and LEONHARDT, P. H. (1976) Enhancement of $rI_n:rC_n$-induced interferon production by amphotericin B. *Antimicrob. Ag. Chemother.* **9**: 551–553.

BORDEN, E. C. and MURPHY, F. A. (1971) The interferon refractory state: *in vivo* and *in vitro* studies of its mechanism. *J. Immunol.* **106**: 134–142.

BORDEN, E. C., PROCHOWNIK, E. V. and CARTER, W. A. (1975) The interferon refractory state. II. Biological characterization of a refractoriness-inducing protein. *J. Immunol.* **114**: 752–756.

BORECKY, L., FUCHSBERGER, N., ZEMLA, J. and LACKOVIC, V. (1971) Properties of the interferon-like virus inhibitor released during interaction of mouse sensitized lymphocytes with their target cells. *Eur. J. Immunol.* **2**: 213–218.

BORECKY, L., LACKOVIC, V., FUCHSBERGER, N. and HAJNICKA, V. (1973) Stimulation of interferon production in macrophages. In: *Activation of Macrophages*, pp. 111–122. Excerpta Medica International Congress Series No. 325. Schloss Reisensburg, Germany.

BORECKY, L., LACKOVIC, V. and WASCHKE, K. (1968) Enhancing effect of anti-endo-toxic serum on interferon induction in mouse peritoneal cells by endo-toxin. *Acta Virol.* **12**: 551–553.

BOUSQUET, C., SENGLAT, C. and RAMUZ, M. (1976) Localisation de l'inducteur d'interferon chez *Brucella suis*. *Compt. Rend. Acad. Sci.* (*Paris*) **282**: 513–516.

BRAUN, W., REGELSON, W., YAJIMA, Y. and ISHIZUKA, M. (1970) Stimulation of antibody formation by pyran copolymer. *Proc. Soc. exp. Biol. Med.* **133**: 171–175.

BREINIG, M. C., ARMSTRONG, J. A. and HO, M. (1975) Rapid onset of hyporesponsiveness to interferon induction on re-exposure to polyribonucleotide. *J. gen. Virol.* **26**: 149–158.

BRESLOW, D. S., EDWARDS, E. I. and NEWBURG, N. R. (1973) Divinyl ether-maleic anhydride (pyran) copolymer used to demonstrate the effect of molecular weight on biological activity. *Nature* **246**: 160–162.

BRITTEN, R. J. and DAVIDSON, E. H. (1969) Gene regulation for higher cells: a theory. *Science* **165**: 349–357.

BROWN, G. E., LEBLEU, B., KAWAKITA, M., SHAILA, S., SEN, G. C. and LENGYEL, P. (1976) Increased endonuclease activity in an extract from mouse Ehrlich ascites tumor cells which had been treated with a partially purified interferon preparation: dependence on double-stranded RNA. *Biochem. biophys. Res. Commun.* **69**: 114–122.

BUCK, K. W., CHAIN, E. B. and HIMMELWEIT, F. (1971) Comparison of interferon induction in mice by purified *penicillium chrysogenum* virus and derived double-stranded RNA. *J. gen. Virol.* **12**: 131–139.

BUCKLER, C. E., DU BUY, H. G., JOHNSON M. C. and BARON, S. (1971) Kinetics of serum interferon response in mice after single and multiple injections of polyI·polyC. *Proc. Soc. exp. Biol. Med.* **136**: 394–398.

BURKE, D. C. (1973) The mechanisms of interferon formation. In: *Interferons and Interferon Inducers*, pp. 107–133, FINTER, N. B. (ed.) North-Holland Publishing Co., Amsterdam.

BURKE, D. C. and ISAACS, A. (1958) Some factors affecting the production of interferon. *Br. J. exp. Path.* **39**: 452–458.

CAME, P. E., LIEBERMAN, M., PASCALE, A. and SHIMONASKI, G. (1969) Antiviral activity of an interferon-inducing synthetic polymer. *Proc. Soc. exp. Biol. Med.* **131**: 443–446.

CANTELL, K. and PAUCKER, K. (1963) Quantitative studies on viral interference in suspended L cells. IV. Production and assay of interferon. *Virology* **21**: 11–21.

CANTOR, H., ASOFSKY, R. and LEVY, H. B. (1970) The effect of polyinosinic–polycytidylic acid upon graft-vs-host activity in BALB/c mice. *J. Immunol.* **104**: 1035–1038.
CARTER, W. A. and DE CLERCQ, E. (1974) Viral infection and host defense. *Science* **186**: 1172–1178.
CARTER, W. A., O'MALLEY, J., BEESON, M., CUNNINGTON, P., KELVIN, A., VERE-HODGE, A., ALDERFER, J. L. and TSO, P. O. P. (1976) An integrated and comparative study of the antiviral effects and other biological properties of the polyinosinic·polycytidylic acid duplex and its mismatched analogues. III. Chronic effects and immunological features. *Mol. Pharmac.* **12**: 440–453.
CARTER, W. A., PITHA, P. M., MARSHALL, L. W., TAZAWA, I., TAZAWA, S. and TSO P. O. P. (1972) Structural requirements of the $rI_n \cdot rC_n$ complex for induction of human interferon. *J. molec. Biol.* **70**: 567–587.
CELMA, M. L. and EHRENFELD, E. (1974) Effect of poliovirus double-stranded RNA on viral and host-cell protein synthesis. *Proc. natn. Acad. Sci. USA* **71**: 2440–2444.
CENTIFANTO, Y. M., GOORHA, R. M. and KAUFMAN, H. E. (1970) Interferon induction in rabbit and human tears. *Am. J. Ophthalmol.* **70**: 1006–1009.
CEROTTINI, J. C., BRUNNER, K. T., LINDAHL, P. and GRESSER, I. (1973) Inhibitory effect of interferon preparations and inducers on the multiplication of transplanted allogeneic spleen cells and syngeneic bone marrow cells. *Nature New Biol.* **242**: 152–153.
CHADHA, K. C., DAVEY, M. W., BYRD, D. M. and CARTER, W. A. (1974) Differential production of interferon and refractoriness inducing principle in L cells. *Infect. Immunity* **10**: 1057–1061.
CHANDRA, P., WILL, G., GERICKE, D. and GÖTZ, A. (1974) Inhibition of DNA polymerases from RNA tumor viruses by tilorone and congeners: site of action. *Biochem. Pharmac.* **23**: 3259–3265.
CHANDRA, P. and WOLTERSDORF, M. (1974) Influence of tilorone and congeners on the secondary structure and template activity of DNA. *FEBS Lett.* **41**: 169–173.
CHANDRA, P. and WOLTERSDORF, M. (1976) Tilorone hydrochloride—a specific probe for A–T regions of duplex deoxyribonucleic acid. *Biochem. Pharmac.* **25**: 877–880.
CHANDRA, P., ZUNINO, F., GAUR, V. P., ZACCARA, A., WOLTERSDORF, M., LUONI, G. GÖTZ, A. (1972c) Mode of tilorone hydrochloride interaction to DNA and polydeoxyribonucleotides. *FEBS Lett.* **28**: 5–9.
CHANDRA, P., ZUNINO, F. and GÖTZ, A. (1972a) Bis-DEAE-fluorenone: a specific inhibitor of DNA polymerases from RNA tumor viruses. *FEBS Lett.* **22**: 161–164.
CHANDRA, P., ZUNINO, F., ZACCARA, A., WACKER, A. and GÖTZ, A. (1972b) Influence of tilorone hydrochloride on the secondary structure and template activity of DNA. *FEBS Lett.* **23**: 145–148.
CHANY, C. and VIGNAL, M. (1970) Effect of prolonged interferon treatment on mouse embryonic fibroblasts transformed by murine sarcoma virus. *J. gen. Virol.* **7** 203–210.
CHAO, J., CHAO, L. and SPEYER, J. (1971) The inhibition of f2 RNA directed *E. Coli* protein synthesis by poly rI·rC *in vitro*. *Biochem. biophys. Res. Commun.* **45**: 1096–1102.
CHESTER, T. A. DE CLERCQ, E. and MERIGAN, T. C. (1971) Effect of separate and combined injections of poly rI·poly rC and endotoxin on reticuloendothelial activity, interferon, and antibody production in the mouse. *Infect. Immunity* **3**: 516–520.
CHRETIEN, P. B., MATTHEWS, JR., W. and TWOMEY, P. L. (1973) Serum ribonucleases in cancer: relation to tumor histology. *Cancer* **31**: 175–179.
CLAES, P., BILLIAU, A., DE CLERCQ, E., DESMYTER, J., SCHONNE, E., VANDERHAEGHE, H. and DE SOMER, P. (1970) Polyacetal carboxylic acids: a new group of antiviral polyanions. *J. Virol.* **5**: 313–320.
CLAVELL, L. A. and BRATT, M. A. (1971) Relatship between the ribonucleic acid synthesizing capacity of ultraviolet-irradiated Newcastle Disease Virus and its ability to induce interferon. *J. Virol.* **8**: 500–508.
COLBY, C. (1971) The induction of interferon by natural and synthetic polynucleotides. *Prog. Nucleic Acid. Res. Mol. Biol.* **11**: 1–32.
COLBY, C. and CHAMBERLIN, M. J. (1969) The specificity of interferon induction in chick embryo cells by helical RNA. *Proc. natn. Acad. Sci. USA* **63**: 160–167.
COLBY, C. and DUESBERG, P. H. (1969) Double-stranded RNA in vaccinia virus infected cells. *Nature* **222**: 940–944.
COLBY, C. and MORGAN, M. J. (1971) Interferon induction and action. *A. Rev. Microbiol.* **25**: 333–360.
COLBY, C., STOLLAR, B. D. and SIMON, M. I. (1971) Interferon induction: DNA–RNA hybrid or double-stranded RNA? *Nature New Biol.* **229**: 172–174.
COLE, B. C., OVERALL, J. C., JR., LOMBARDI, P. S. and GLASGOW, L. A. (1976) Induction of interferon in ovine and human lymphocyte cultures by mycoplasmas. *Infect. Immunity* **14**: 88–94.
COLLAVO, D., FINCO, B. and CHIECO-BIANCHI, L. (1972) Immune reactivity following polyI·polyC treatment in mice. *Nature New Biol.* **239**: 154–155.
COLLINS, F. M. (1975) Effect of tilorone treatment on intracellular microbial infections in specific-pathogen-free mice. *Antimicrob. Ag. Chemother.* **7**: 447–452.
CORDELL-STEWART, B. and TAYLOR, M. W. (1971) Effect of double-stranded viral RNA on mammalian cells in culture. *Proc. natn. Acad. Sci. USA* **68**: 1326–1330.
CORDELL-STEWART, B. and TAYLOR, M. W. (1973a) Effect of viral double-stranded RNA on protein synthesis in intact cells. *J. Virol.* **11**: 232–237.
CORDELL-STEWART, B. and TAYLOR, M. W. (1973b) Effect of viral double-stranded RNA on mammalian cells in culture: cytotoxicity under conditions preventing viral replication and protein synthesis. *J. Virol.* **12**: 360–366.
CRENSHAW, R. R., LUKE, G. M. and SIMINOFF, P. (1976) Interferon inducing activities of derivatives of 1,3-dimethyl-4-(3-dimethylaminopropylamino)-1H-pyrazolo[3,4-*b*]quinoline and related compounds. *J. Med. Chem.* **19**: 262–275.
CRICK, F. (1971) General model for the chromosomes of higher organisms. *Nature* **234**, 25–27.
D'ALESSIO, G., ZOFRA, S. and LIBONATI, M. (1972) Action of dimeric ribonucleases on double-stranded RNA. *FEBS Lett.* **24**: 355–358.
DARNBROUGH, C., HUNT, T. and JACKSON, R. J. (1972) A complex between met-$tRNA_F$ and native 40 S

subunits in reticulocyte lysates and its disappearance during incubation with double-stranded RNA. *Biochem. biophys. Res. Commun.* **48**: 1556–1564.

DEAN, J. H., WALLEN, W. C. and LUCAS, D. O. (1972) Polyinosinic·polycytidylic acid activation of mouse spleen cells *in vitro*. *Nature New Biol.* **237**: 218–219.

DE CLERCQ, E. (1972) Hyporeactivity to interferon production by double-stranded RNA associated with hyporeactivity to antiviral protection and hyporeactivity to toxicity. *Proc. Soc. exp. Biol. Med.* **141**: 340–345.

DE CLERCQ, E. (1973) Nonpolynucleotide interferon inducers. In: *Selective Inhibitors of Viral Functions*, pp. 177–198, CARTER, W. A. (ed.), CRC Press, Cleveland, Ohio.

DE CLERCQ, E. (1974) Synthetic interferon inducers. *Topics in Current Chemistry* **52**: 173–208.

DE CLERCQ, E. (1976) Polynucleotides as inducers of interferon. In: *Structure and Properties of Biopolymers*. Proceedings of the First Cleveland Symposium on Macromolecules held at the Case Western Reserve University, Cleveland, Ohio, pp. 217–243, WALTON A. G. (ed.), North-Holland/Elsevier/Excerpta Medica, Amsterdam.

DE CLERCQ, E., BILLIAU, A., HOBBS, J., TORRENCE, P. F. and WITKOP, B. (1975f) Inhibition of oncornavirus functions by 2'-azido polynucleotides. *Proc. natn. Acad. Sci. USA* **72**: 284–288.

DE CLERCQ, E., BILLIAU, A., TORRENCE, P. F., WATERS, J. A. and WITKOP, B. (1975a) Antiviral and antimetabolic activities of poly(7-deazaadenylic acid) and poly-(7-deazainosinic acid). *Biochem. Pharmac.* **24**: 2233–2238.

DE CLERCQ, E. and DE SOMER, P. (1968a) Protective effect of interferon and polyacrylic acid in newborn mice infected with a lethal dose of vesicular stomatitis virus. *Life Sci.* **7**: 925–933.

DE CLERCQ, E. and DE SOMER, P. (1968b) Effect of interferon, polyacrylic acid and polymethacrylic acid on tail lesions in mice infected with vaccinia virus. *Appl. Microbiol.* **16**: 1314–1319.

DE CLERCQ, E. and DE SOMER, P. (1969) Prolonged antiviral protection by interferon inducers. *Proc. Soc. exp. Biol. Med.* **132**: 699–703.

DE CLERCQ, E. and DE SOMER, P. (1971a) Antiviral activity of polyribocytidylic acid in cells primed with polyriboinosinic acid. *Science* **173**: 260–262.

DE CLERCQ, E. and DE SOMER, P. (1971b) Comparative study of the efficacy of different forms of interferon therapy in the treatment of mice challenged intranasally with vesicular stomatitis virus. *Proc. Soc. exp. Biol. Med.* **138**: 301–307.

DE CLERCQ, E. and DE SOMER, P. (1972a) Mechanism of the antiviral activity resulting from sequential administration of complementary homopolyribonucleotides to cell cultures. *J. Virol.* **9**: 721–731.

DE CLERCQ, E. and DE SOMER, P. (1972b) Production of interferon in rabbit cell cultures by mouse L cell-bound poly(rI)·poly(rC). *J. gen. Virol.* **16**: 435–439.

DE CLERCQ, E. and DE SOMER, P. (1973a) Protection of rabbits against local vaccinia virus infection by *brucella abortus* and polyacrylic acid in the absence of systemic interferon production. *Infect. Immunity* **8**: 669–673.

DE CLERCQ, E. and DE SOMER, P. (1973b) Relationship between cell-interaction and antiviral activity of polyriboinosinic acid-polyribocytidylic acid in different cell cultures. *J. gen. Virol.* **19**: 113–123.

DE CLERCQ, E. and DE SOMER, P. (1974) Interferon production in rabbit kidney cell cultures exposed to poly(I)·poly(C) adsorbed to rabbit red blood cells. *J. gen. Virol.* **22**: 271–275.

DE CLERCQ, E. and DE SOMER, P. (1975) Cytotoxicity and interferon inducing activity of poly(I)·poly(C) invariably linked in interferon-treated L cells? *J. gen. Virol.* **27**: 35–44.

DE CLERCQ, E., ECKSTEIN, F. and MERIGAN, T. C. (1969) Interferon induction increased through chemical modification of a synthetic polynucleotide. *Science* **165**: 1137–1139.

DE CLERCQ, E., ECKSTEIN, F. and MERIGAN, T. C. (1970a) Structural requirements for synthetic polyanions to act as interferon inducers. *Ann. N.Y. Acad. Sci.* **173**: 444–461.

DE CLERCQ, E., ECKSTEIN, F., STERNBACH, H. and MERIGAN, T. C. (1970b) The antiviral activity of thiophosphate-substituted polyribonucleotides *in vitro* and *in vivo*. *Virology* **42**: 421–428.

DE CLERCQ, E., ECKSTEIN, F., STERNBACH, H. and MERIGAN, T. C. (1970c) Interferon induction by, and ribonuclease sensitivity of thiophosphate-substituted polyribonucleotides. *Antimicrob. Ag. Chemother.* **1969**, 187–191.

DE CLERCQ, E., EDY, V. G. and CASSIMAN, J. J. (1975b) Non-antiviral activities of interferon are not controlled by chromosome 21. *Nature* **256**: 132–134.

DE CLERCQ, E., EDY, V. G., TORRENCE, P. F., WATERS, J. A. and WITKOP, B. (1976a) *In vitro* and *in vivo* antiviral activity of poly(7-deazainosinic acid) derived complexes. *Mol. Pharmac.* **12**: 1045–1051.

DE CLERCQ, E., HATTORI, M. and IKEHARA, M. (1975c) Antiviral activity of polynucleotides: copolymers of inosinic acid and N2-dimethylguanylic or 2-methylthioinosinic acid. *Nucleic Acids Res.* **2**: 121–129.

DE CLERCQ, E. and JANIK, B. (1973) Antiviral activity of polynucleotides: poly(2'-fluoro-2'-deoxyuridylic acid). *Biochim. biophys. Acta* **324**, 50–56.

DE CLERCQ, E., JANIK, B. and SOMMER, R. G. (1976b) Biological, biochemical and physiochemical evidence for the existence of the polyadenylate·polyuridylate·poly2'-fluoro-2'-deoxyuridylate triple stranded complex. *Chem. Biol. Interactions* **14**: 113–125.

DE CLERCQ, E., JANIK, B. and SOMMER, R. G. (1977) Effects of ionenes on interferon induction by poly(inosinic acid)·poly(cytidylic acid). *Antimicrob. Ag. Chemother.* **11**: 756–759.

DE CLERCQ, E. and LUCZAK, M. (1976a) Intranasal challenge of mice with herpes simplex virus: an experimental model for evaluation of the efficacy of antiviral drugs. *J. Infect. Dis.* **133** (supplement): A226–A236.

DE CLERQ, E. and LUCZAK, M. (1976b) Antiviral activity of carbopol, a cross-linked polycarboxylate. *Arch. Virol.* **52**: 151–158.

DE CLERCQ, E. and MERIGAN, T. C. (1969a) Requirement of a stable secondary structure for the antiviral activity of polynucleotides. *Nature*, **222**: 1148–1152.

DE CLERCQ, E. and MERIGAN, T. C. (1969b) Local and systemic protection by synthetic polyanionic interferon inducers in mice against intranasal vesicular stomatitis virus. *J. gen. Virol.* **5**: 359–368.

De Clercq, E. and Merigan, T. C. (1969c) An active interferon inducer obtained from *hemophilus influenzae* type B. *J. Immunol.* **103**: 899–906.

De Clercq, E. and Merigan, T. C. (1970) Stimulation or inhibition of interferon production depending on time of cycloheximide administration. *Virology* **42**: 799–802.

De Clercq, E. and Merigan, T. C. (1971a) Thermal activation of the antiviral activity of synthetic polyribonucleotides: influence of DEAE-dextran in various cell cultures. *J. gen. Virol.* **10**: 125–130.

De Clercq, E. and Merigan, T. C. (1971b) Bis-DEAE-fluorenone: mechanism of antiviral protection and stimulation of interferon production in the mouse. *J. Infect. Dis.* **123**: 190–199.

De Clercq, E., Nuwer, M. R. and Merigan, T. C. (1970d) The role of interferon in the protective effect of a synthetic double-stranded polyribonucleotide against intranasal vesicular stomatitis virus challenge in mice. *J. clin. Invest.* **49**: 1565–1577.

De Clercq, E., Rottman, F. M. and Shugar, D. (1974a) Antiviral activity of polynucleotides: poly2′-*O*-ethyl-adenylic acid and poly2′-*O*-ethyluridylic acid. *FEBS Lett.* **42**: 331–334.

De Clercq, E. and Stewart, W. E. II (1973) The breadth of interferon action. In: *Selective Inhibitors of Viral Functions*, pp. 81–106, Carter, W. A. (ed.), CRC Press, Cleveland, Ohio.

De Clercq, E. and Stewart, W. E. II (1974) Integrity of cell-bound poly(I)·poly(C). *J. gen. Virol.* **24**: 201–209.

De Clercq, E., Stewart II, W. E. and De Somer, P. (1972a) Interferon production linked to toxicity of polyriboinosinic acid-polyribocytidylic acid. *Infect. Immunity* **6**: 344–347.

De Clercq, E., Stewart II, W. E. and De Somer, P. (1973a) Poly(rI) more important than poly(rC) in the interferon induction process by poly(rI)·poly(rC). *Virology* **54**: 278–282.

De Clercq, E., Stewart II, W. E. and De Somer, P. (1973b) Studies on the mechanism of the priming effect of interferon on interferon production by cell cultures exposed to poly(rI)·poly(rC). *Infect. Immunity* **8**: 309–316.

De Clercq, E. and Torrence, P. (1977) Comparative study of various double-stranded RNAs as inducers of human interferon. *J. gen. Virol.* (submitted for publication).

De Clercq, E., Torrence, P. F., De Somer, P. and Witkop, B. (1975d) Biological, biochemical and physicochemical evidence for the existence of polyadenylic·polyuridylic·polyinosinic acid triplex. *J. Biol. Chem.* **250**: 2521–2531.

De Clercq, E., Torrence, P. F., Fukui, T. and Ikehara, M. (1976c) Role of purine N-3 in the biologic activities of poly(A) and poly(I). *Nucleic Acids Res.* **3**: 1591–1601.

De Clercq, E., Torrence, P. F. and Witkop, B. (1974b) Interferon induction by synthetic polynucleotides: importance of purine N-7 and strandwise rearrangement. *Proc. natn. Acad. Sci. USA* **71**: 182–186.

De Clercq, E., Torrence, P. F. and Witkop, B. (1976d) Polynucleotide displacement reactions: detection by interferon induction. *Biochemistry* **15**: 717–724.

De Clercq, E., Torrence, P. F., Witkop B. and De Somer, P. (1975e) Interferon induction by synthetic polynucleotides. Competition between inactive and active polymers. In: *Effects of Interferon On Cells, Viruses and the Immune System*, pp. 215–236, Geraldes, A. (ed.). Academic Press, New York.

De Clercq, E., Torrence, P. F., Witkop, B., Stewart, W. E. II and De Somer, P. (1974c) Interferon induction: tool for establishing interactions among homopolyribonucleotides. *Science* **186**: 835–837.

De Clercq, E., Wells, R. D., Grant, R. C. and Merigan, T. C. (1971) Thermal activation of the antiviral activity of synthetic double-stranded polynucleotides. *J. molec. Biol.* **50**: 83–100.

De Clercq, E., Wells, R. D. and Merigan, T. C. (1970e) Increase in antiviral activity of polynucleotides by thermal activation. *Nature* **226**: 364.

De Clercq, E., Wells, R. D. and Merigan, T. C. (1972b) Studies on the antiviral activity and cell interaction of synthetic double-stranded polyribo- and polydeoxyribonucleotides. *Virology* **47**: 405–415.

De Clercq, E., Zmudzka, B. and Shugar, D. (1972c) Antiviral activity of polynucleotides: role of the 2′-hydroxyl and a pyrimidine 5-methyl. *FEBS Lett.* **24**: 137–140.

Degre, M. (1973) Influence of polyinosinic:polycytidylic acid on the circulating white blood cells in mice. *Proc. Soc. exp. Biol. Med.* **142**: 1087–1091.

De Maeyer, E., De Maeyer-Grignard, J. and Montagnier, L. (1971) Double-stranded RNA from rat liver induces interferon in rat cells. *Nature New Biol.* **229**: 109–110.

De Maeyer-Guignard, J. (1972) Mouse leukemia: depression of serum interferon production. *Science* **177**: 797–798.

De Maeyer-Guignard, J., De Maeyer, E. and Montagnier, L. (1972) Interferon messenger RNA translation in heterologous cells. *Proc. natn. Acad. Sci. USA* **69**: 1203–1207.

Dennis, A. J., Wilson, H. E., Barker, A. D. and Rheins, M. S. (1972) Interferon induction in normal and leukemic human lymphocyte cultures by tilorone hydrochloride. *Proc. Soc. exp. Biol. Med.* **141**: 782–785.

Desmyter, J., Rawls, W. E. and Melnick, J. L. (1968) A human interferon that crosses the species line. *Proc. natn. Acad. Sci. USA* **59**: 69–76.

Desmyter, J., Ray, M. B., De Groote, J., Bradburne, A. F., Desmet, V. J., Edy, V. V. G., Billiau, A., De Somer, P. and Mortelmans, J. (1976) Administration of human fibroblast interferon in chronic hepatitis-B infection. *Lancet* **ii**: 645–646.

Desmyter, J. and Stewart II, W. E. (1976) Molecular modification of interferon: attainment of human interferon in a conformation active on cat cells but inactive on human cells. *Virology* **70**: 451–458.

De Somer, P. and Billiau, A. (1966) Interferon production by the spleen of rats after intravenous injection of sindbis virus or heat-killed *Escherichia coli*. *Arch. Ges. Virusforsch.* **XIX**: 143–154.

De Somer, P., De Clercq, E., Billiau, A., Schonne, E. and Claesen, M. (1968a) Antiviral activity of polyacrylic and polymethacrylic acids. *J. Virol.* **2**: 878–885.

De Somer, P., De Clercq, E., Billiau, A., Schonne, E. and Claesen, M. (1968b) Antiviral activity of polyacrylic and polymethacrylic acids II. Mode of action *in vivo*. *J. Virol.* **2**: 886–893.

De Somer, P., De Clercq, E., Cocito, C. and Billiau, A. (1970) The interferon inducer from Brucella. *Ann. N.Y. Acad. Sci.* **173**: 274–281.

DE VITA, V., CANELLOS, G., CARBONE, P., BARON, S., LEVY, H. and GRALNICK, H. (1970) Clinical trials with the interferon (InF) inducer polyinosiniccytidylic (PIC). *Proc. Am. Ass. Cancer Res.* **11**: 21.

DIAMANTSTEIN, T. (1973) Stimulation of humoral immune response by tilorone hydrochloride. *Immunology* **24**: 771–775.

DIANZANI, F., BARON, S., BUCKLER, C. E. and LEVY, H. B. (1971) Mechanisms of DEAE-dextran enhancement of polynucleotide induction of interferon. *Proc. Soc. exp. Biol. Med.* **136**: 1111–1114.

DIANZANI, F., CANTAGALLI, P., GAGNONI, S. and RITA, G. (1968) Effect of DEAE-dextran on production of interferon induced by synthetic double-stranded RNA in L cell cultures. *Proc. Soc. exp. Biol. Med.* **128**: 708–710.

DIANZANI F., GAGNONI, S., BUCKLER, C. E. and BARON, S. (1970) Studies of the induction of interferon system by nonreplicating Newcastle Disease Virus. *Proc. Soc. exp. Biol. Med.* **133**: 324–328.

DIANZANI, F., PUGLIESE, A. and BARON, S. (1974) Induction of interferon by nonreplicating single-stranded RNA virus. *Proc. Soc. exp. Biol. Med.* **145**: 428–433.

DIANZANI, F., RITA, G., CANTAGALLI, P. and GAGNONI, S. (1969) Effect of DEAE-dextran on interferon production and protective effect in mice treated with double-stranded polynucleotide complex poly-inosinic-polycytidylic acid. *J. Immunol.* **102**: 24–27.

DIEDERICH, J., LODEMANN, E. and WACKER, A. (1972) New type of interferon inducer. *Die Naturwissenschaften* **4**: 172–173.

DIEDERICH, J., LODEMANN, E. and WACKER, A. (1973) Basic dyes as inducers of interferon-like activity in mice. *Arch. Ges. Virusforsch.* **40**: 82–86.

DOSKOCIL, J., FUCHSBERGER, N., VETRAK, J., LACKOVIC, V. and BORECKY, L. (1971) Double-stranded f_2 phage RNA as interferon inducer. *Acta Virol.* **15**: 523.

DOUGLAS, JR., R. G. and BETTS, R. F. (1974) Effect of induced interferon in experimental rhinovirus infections in volunteers. *Infect. Immunity* **9**: 506–510.

DOUGLAS, R. G., JR., BETTS, R. F., SIMONS, R. L., HOGAN, P. W. and ROTH, F. K. (1975) Evaluation of a topical interferon inducer in experimental influenza infection in volunteers. *Antimicrob. Ag. Chemother.* **8**: 684–687.

DUBOVI, E. J. and AKERS, T. G. (1972) Interferon induction by Colorado tick fever virus: a double-stranded RNA virus. *Proc. Soc. exp. Biol. Med.* **139**: 123–127.

DU BUY, H. G., JOHNSON, M. L., BUCKLER, C. E. and BARON, S. (1970) Relationship between dose size and dose interval of polyinosinic-polycyticylic acid and interferon hyporesponsiveness in mice. *Proc. Soc. exp. Biol. Med.* **135**: 340–344.

EDY, V. G., SZEKELY, M., HIMMILWEIT, F. and CHAIN, E. B. (1974) Effect of fragmentation on interferon induction by double-stranded virus RNA. *J. gen. Virol.* **23**: 191–195.

EHRENFELD, E. and HUNT, T. (1971) Double-stranded poliovirus RNA inhibits initiation of protein synthesis by reticulocyte lysates. *Proc. natn. Acad. Sci. USA* **68**: 1075–1078.

EIGNER, J. and DOTY, P. (1965) The native, denatured and renatured states of deoxyribonucleic acid. *J. molec. Biol.* **12**: 549–580.

ENGLANDER, J. J., KALLENBACH, N. R. and ENGLANDER, S. W. (1972) Hydrogen exchange study of some polynucleotides and transfer RNA. *J. molec. Biol.* **63**: 153–169.

EPSTEIN, L. B. (1976) The ability of macrophages to augment *in vitro* mitogen- and antigen-stimulated production of interferon and other mediators of cellular immunity by lymphocytes. In: *Immunobiology of the Macrophage.* pp. 201–234, Academic Press, New York, San Francisco, London.

EPSTEIN, L. B. and AMMANN, A. J. (1974) Evaluation of T lymphocyte effector function in immunodeficiency diseases: abnormality in mitogen-stimulated interferon in patients with selective IgA deficiency. *J. Immunol.* **112**: 617–626.

EPSTEIN, L. B. and CLINE, M. J. (1974) Chronic lymphocytic leukaemia. Studies on mitogen-stimulated lymphocyte interferon as a new technique for assessing T lymphocyte effector function. *Clin. Exp. Immunol.* **16**: 553–563.

EPSTEIN, L. B., CLINE, M. J. and MERIGAN, T. C. (1971) The interaction of human macrophages and lymphocytes in the phytohemagglutinin-stimulated production of interferon. *J. clin. Invest.* **50**: 744–753.

EPSTEIN, L. B., CLINE, M. J. and MERIGAN, T. C. (1972b) The *in vitro* interaction of immune macrophages and lymphocytes in the antigen (PPD)-induced production of interferon. In: *Proceedings of the Sixth Leucocyte Culture Conference*, pp. 265–282. Academic Press, New York and London.

EPSTEIN, L. B., KRETH, H. W. and HERZENBERG, L. A. (1974) Fluorescence-activated cell sorting of human T and B lymphocytes. II. Identification of the cell type responsible for interferon production and cell proliferation in response to mitogens. *Cell. Immunol.* **12**: 407–421.

EPSTEIN, L. B., STEVENS, D. A. and MERIGAN, T. C. (1972a) Selective increase in lymphocyte interferon response to vaccinia antigen after revaccination. *Proc. natn. Acad. Sci. USA* **69**: 2632–2636.

FALCOFF, R. (1972) Some properties of virus and immune-induced human lymphocyte interferons. *J. gen. Virol.* **16**: 251–253.

FALCOFF, R. and FALCOFF, E. T. (1969) Induction de la synthèse d'intérferon par des RNA bicaténaires. I. Application à l'étude du cycle de multiplication du virus mengo. *Biochim. biophys. Acta* **182**: 501–510.

FALCOFF, R. and FALCOFF, E. (1970) Induction de la synthèse d'interférón par des RNA bicaténaires. II. Etude de la forme intermédiaire de réplication du virus mengo. *Biochim. biophys. Acta* **199**: 147–158.

FALCOFF, E., FALCOFF, R., CATINOT, L., DE VOMECOURT, A. and SANCEAU, J. (1972a) Synthesis of interferon in human lymphocytes stimulated *'in vitro'* by antilymphocytic serum. *Eur. J. Clin. Biol. Res.* **XVII**: 20–26.

FALCOFF, E., FALCOFF, R., CHERBY, J., FLORENT, J., LUNEL, J., NINET, L., DE RATULD, Y., TISSIER, R., VUILLEMIN, B. and WERNER, G. H. (1973) Double-stranded ribonucleic acid from mengo virus: production, characterization, and interferon-inducing and antiviral activities in comparison with polyriboinosinic-polyribocytidylic acid. *Antimicrob. Ag. Chemother.* **3**: 590–598.

FALCOFF, R., ORIOL, R. and ISCAKI, S. (1972b) Lymphocyte stimulation and interferon induction by 7 S anti-human lymphocyte globulins and their uni- and divalent fragments. *Eur. J. Immunol.* **2**: 476–478.

FALCOFF, E. and PEREZ-BERCOFF, R. (1969) Interferons humains induits *in vitro* par des polynucleotides synthetiques. *Biochim. biophys. Acta* **174**: 108–116.

FEINGOLD, D. S., KELETI, G. and YOUNGNER, J. S. (1976) Antiviral activity of *Brucella abortus* preparations: separation of active components. *Infect. Immunity* **13**: 763–767.

FEINGOLD, D. S., YOUNGNER, J. S. and CHEN, J. (1968) Interferon production in mice by cell wall mutants of *Salmonella typhimurium*. II. Effect of purified glycolipids from S and Re chemotypes. *Biochem. biophys. Res. Commun.* **32**: 554–557.

FEINGOLD, D. S., YOUNGNER, J. S. and CHEN, J. (1970) Interferon production in mice by cell wall mutants of *Salmonella typhimurium*. III. Role of lipid moiety of bacterial lipopolysaccharide in interferon production in animals. *Ann. N.Y. Acad. Sci.* **173**: 249–254.

FELSENFELD, G. and MILES, H. T. (1969) The physical and chemical properties of nucleic acids. *A. Rev. Biochem.* **36**: 407–448.

FIELD, A. K. (1973) Interferon induction by polynucleotides. In: *Selective Inhibitors of Viral Functions*, pp. 149–176, CARTER, W. A. (ed.), CRC Press, Cleveland, Ohio.

FIELD, A. K., LAMPSON, G. P., TYTELL, A. A. and HILLEMAN, M. R. (1972a) Demonstration of double-stranded ribonucleic acid in concentrates of RNA viruses. *Proc. Soc. exp. Biol. Med.* **141**: 440–444.

FIELD, A. K., LAMPSON, G. P., TYTELL, A. A., NEMES, M. M. and HILLEMAN, M. R. (1967a) Inducers of interferon and host resistance, IV. Double-stranded replicative form RNA (MS2-RF-RNA) from *E. coli* infected with MS2 coliphage. *Proc. natn. Acad. Sci. USA* **58**: 2102–2108.

FIELD, A. K., TYTELL, A. A. and HILLEMAN, M. R. (1968) Cellular uptake of rI:rC and the *in vitro* production of cell-associated interferon. *Bacteriol. Proc.* **1969**: 150.

FIELD, A. K., TYTELL, A. A., LAMPSON, G. P. and HILLEMAN, M. R. (1968) Inducers of interferon and host resistance. V. *In Vitro* studies. *Proc. natn. Acad. Sci. USA* **61**: 340–346.

FIELD, A. K., TYTELL, A. A., LAMPSON, G. P. and HILLEMAN, M. R. (1967b) Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc. natn. Acad. Sci. USA* **58**: 1004–1009.

FIELD, A. K., TYTELL, A. A., LAMPSON, G. P. and HILLEMAN, M. R. (1972b) Cellular control of interferon production and release after treatment with poly I:C inducer. *Proc. Soc. exp. Biol. Med.* **140**: 710–714.

FIELD, A. K., YOUNG, C. W., KRAKOFF, I. H., TYTELL, A. A., LAMPSON, G. P., NEMES, M. M. and HILLEMAN, M. R. (1971) Induction of interferon in human subjects by poly I:C. *Proc. Soc. exp. Biol. Med.* **136**: 1180–1186.

FINK, K., ADAMS, W. S. and SKOOG, W. A. (1971) Serum ribonuclease in multiple myeloma. *Am. J. Med.* **50**: 450–457.

FINKELSTEIN, M. S., BAUSEK, G. H. and MERIGAN, T. C. (1968) Interferon inducers *in vitro*: difference in sensitivity to inhibitors of RNA and protein synthesis. *Science* **161**: 465–468.

FINTER, N. B. (ed.) (1973) *Interferons and Interferon Inducers*. American Elsevier Publishing Co., New York.

FLIKKE, M., KJELDSBERG, E. and LAHELLE, O. (1970) Interference with cell viability and poliovirus multiplication by polyinosinic-polycytidylic acid. *J. gen. Virol.* **9**: 111–117.

FOLAYAN, J. O. and HUTCHINSON, D. W. (1974) Poly(5-fluorocytidylic acid). *Biochim. biophys. Acta* **340**: 194–198.

FOX, C. F. and KEITH, A. (1972) *Membrane Molecular Biology*. Sinauer Associates, Stanford, Connecticut.

FRAYSSINET, C., GRESSER, I., TOVEY, M. and LINDAHL, P. (1973) Inhibitory effect of potent interferon preparations on the regeneration of mouse liver after partial hepatectomy. *Nature* **245**: 146–147.

FRESCO, J. R. and MASSOULIE, J. (1963) Polynucleotides. V. Helix-coil transition of polyriboguanylic acid. *J. Am. Chem. Soc.* **85**: 1352–1353.

FRIEDMAN, R. M. (1966) Effect of interferon treatment on interferon production. *J. Immunol.* **96**: 872–877.

FRIEDMAN, R. M. and COOPER, H. L. (1967) Stimulation of interferon production in human lymphocytes by mitogens. *Proc. Soc. exp. Biol. Med.* **125**: 901–905.

FRIEDMAN, H. M., JOHNSON, A. G. and PAN, P. (1969) Stimulatory effect of polynucleotides on short term leukocyte cultures. *Proc. Soc. exp. Biol. Med.* **132**: 916–918.

GAJDOSOVA, E., DOSKOCIL, J. and MAYER, V. (1973) Dependence of antiviral activity on molecular weight in an interferon stimulator of the natural double-stranded RNA type. *Acta Virol.* **17**: 196–202.

GALABOV, A. S. and GALABOV, S. M. (1973) Interferon induction by detoxicated bacterial endotoxins. *Acta Virol.* **17**: 493–500.

GANDHI, S. S. and BURKE, D. C. (1970) Interferon production by myxoviruses in chick embryo cells. *J. gen. Virol.* **6**: 95–103.

GANDHI, S. S., BURKE, D. C. and SCHOLTISSEK, C. (1970) Virus RNA synthesis by ultraviolet-irradiated Newcastle disease virus and interferon production. *J. gen. Virol.* **9**: 97–99.

GANTI, T., MECS, I. and KOTAI, A. (1976) Potentiation of poly I:C induced viral resistance by polycationic modified polypeptides. *Arch. virol.* **51**: 227–234.

GATMAITAN, B. G., STANLEY, E. D. and JACKSON, G. G. (1973) The limited effect of nasal interferon induced by rhinovirus and a topical chemical inducer on the course of infection. *J. Infect. Dis.* **127**: 401–407.

GAUNTT, C. J. (1973) Induction of interferon in L cells by reoviruses. *Infect. Immunity* **7**: 711–717.

GIBSON, J. P., MEGEL, H., CAMYRE, K. P. and MICHAEL, J. G. (1976) Effect of tilorone hydrochloride on the lymphoid and interferon responses of athymic mice. *Proc. Soc. exp. Biol. Med.* **151**: 264–266.

GIFFORD, G. E., TIBOR, A. and PEAVY, D. L. (1971) Interferon production in mixed lymphocyte cell cultures. *Infect. Immunity* **3**: 164–166.

GINSBERG, H. S. and HORSFALL, F. L., JR., (1951) Therapy of infection with pneumonia virus of mice (PVM). Effect of a polysaccharide on the multiplication cycle of the virus and on the course of the viral pneumonia. *J. Exp. Med.* **93**: 161–171.

GIRON, D. J., ALLEN, P. T., PINDAK, F. F. and SCHMIDT, J. P. (1971) Interferon-inducing characteristics of MM virus. *Appl. Microbiol.* **21**: 387–393.

GIRON, D. J., SCHMIDT, J. P., BALL, R. J. and PINDAK, F. F. (1972a) Effect of interferon inducers and interferon on bacterial infections. *Antimicrob. Ag. Chemother.* **1**: 80–81.

GIRON, D. J., SCHMIDT, J. P. and PINDAK, F. F. (1972b) Induction of viral resistance by poly I:C in cells which apparently produce no interferon. *Proc. Soc. exp. Biol. Med.* **141**: 47–51.

GIRON, D. J., SCHMIDT, J. P. and PINDAK, F. F. (1972c) Tilorone hydrochloride: lack of correlation between interferon induction and viral protection. *Antimicrob. Ag. Chemother.* **1**: 78–79.

GLASGOW, L. A. (1966) Leukocytes and interferon in the host response to viral infections. II. Enhanced interferon response of leukocytes from immune animals. *J. Bacteriol.* **91**: 2185–2191.

GLAZ, E. T., SZOLGAY, E., STÖGER, I. and TALAS, M. (1973) Antiviral activity and induction of interferon-like substance by quinacrine and acranil. *Antimicrob. Ag. Chemother.* **3**: 537–541.

GLAZ, E. T. and TALAS, M. (1975) Comparison of the activity of small molecular interferon inducers: tilorone and acridine drugs. *Arch. Virol.* **48**: 375–384.

GOLGHER, R. R. and PAUCKER, K. (1973) Blocking of interferon production by chromatographically purified L cell interferon. *Proc. Soc. exp. Biol. Med.* **142**: 167–174.

GOORE, M. Y., DICKSON, J. H., LIPKINS, S. and DI CUOLLO, C. J. (1973) The production of human leukocyte interferon in a serum free medium. *Proc. Soc. exp. Biol. Med.* **142**: 46–49.

GOORHA, R. M. and GIFFORD, G. E. (1970) Interferon induction by heat-inactivated Semliki Forest Virus. *Proc. Soc. exp. Biol. Med.* **134**: 1142–1147.

GOORHA, R. M. and GIFFORD, G. E. (1971) Comparison of interferon induction by active and inactive Semliki Forest Virus. *J. gen. Virol.* **10**: 117–124.

GRAZIADEI III, W. D. and LENGYEL, P. (1972) Translation of *in vitro* reovirus messenger RNA's into proteins of reovirus capsid proteins in a mouse L cell extract. *Biochem. biophys. Res. Commun.* **46**: 1816–1823.

GRAZIANO, K. D., LEVY, C. C., SCHMUKLER, M. and MARDINEY JR., M. R. (1974) Parameters for effective use of synthetic double-stranded polynucleotides in the amplification of immunologically induced lymphocyte tritiated thymidine incorporation. *Cell. Immunol.* **11**: 47–56.

GREEN, J. A., COOPERBAND, S. R. and KIBRICK, S. (1969) Immune specific induction of interferon production in cultures of human blood lymphocytes. *Science* **164**: 1415–1417.

GREEN, M., RANKIN, A., GERARD, G. F., GRANDGENETT, D. P. and GREEN, M. R. (1975) Inhibition of purified DNA polymerase of RNA tumor viruses by fluoranthene derivatives and analogues of tilorone hydrochloride. *J. natn. Cancer Inst.* **55**: 433–442.

GRESSER, I., BANDU, M. T., BROUTY-BOYE, D. and TOVEY, M. (1974) Pronounced antiviral activity of human interferon on bovine and porcine cells. *Nature* **251**: 543–545.

GRILL, L. K., SUN, J. D. and KANDEL, J. (1973) Effect of double-stranded RNA on protein synthesis in an *in vitro* wheat germ embryo system. *Biochem. biophys. Res. Commun.* **73**: 149–151.

GRISAR, J. M., HICKEY, K. R., FLEMING, R. W. and MAYER, G. D. (1974) Bis-basic-substituted polycyclic aromatic compounds. A new class of antiviral agents. 4. Bis-basic sulfonamides of anthraquinone. *J. Med. Chem.* **17**: 890–893.

GROELKE, J. W., CAMYRE, K. P. and MAYER, G. D. (1975) Enhancement of interferon production *in vitro*: a property of tilorone-poly rI:rC/DEAE-dextran. *Proc. Soc. exp. Biol. Med.* **148**: 1044–1047.

GROSSBERG, S. E. (1972) The interferons and their inducers: molecular and therapeutic considerations. *New Engl. J. Med.* **287**: 1319.

GRUENEWALD, R. and LEVINE, S. (1976) Effect of tilorone on susceptibility of mice to primary or secondary infection with *Listeria monocytogenes. Infect. Immunity* **13**: 1613–1618.

GRUNBERG-MANAGO, M. (1963) Polynucleotide phosphorylase. *Prog. Nucleic Acid Res. Molec. Biol.* **1**: 93–133.

GUSCHLBAUER, W. (1972) Why is guanosine different? In: *The Jerusalem Symposium on Quantum Chemistry and Biochemistry. IV. The Purines, Theory and Experiment*, pp. 297–309, BERGMAN, E. D. and PULLMAN, B. (eds.). Academic Press, New York.

HAAHR, S., RASMUSSEN, L. and MERIGAN, T. C. (1976) Lymphocyte transformation and interferon production in human mononuclear cell microcultures for assay of cellular immunity to herpes simplex virus. *Infect. Immunity* **14**: 47–54.

HABER, J., ROSENAU, W. and GOLDBERG, M. (1972) Separate factors in phytohaemagglutinin induce lymphotoxin, interferon, and nucleic acid synthesis. *Nature New Biol.* **238**: 60–61.

HARMON, M. W., JANIS, B. and LEVY, H. B. (1974) Post-exposure prophylaxis of murine rabies with polyinosinic-polycytidylic acid and chlorite-oxidized amylose. *Antimicrob. Ag. Chemother.* **6**: 507–511.

HARPER, H. D. and PITHA, P. M. (1973) Effect of concanavalin A on interferon induction by poly I:C. *Biochem. biophys. Res. Commun.* **53**: 1220–1226.

HASELKORN, R. and FOX, C. F. (1965) Synthesis and properties of a complex of polyriboguanylic acid and polyribocytidylic acid. *J. Mol. Biol.* **13**: 780–790.

HAVELL, E. A., BERMAN, B., OGBURN, C. A., BERG, K., PAUCKER, K. and VILCEK, J. (1975a) Two antigenically distinct species of human interferon. *Proc. natn. Acad. Sci. USA* **72**: 2185–2187.

HAVELL, E. A. and VILCEK, J. (1972) Production of high-titered interferon in cultures of human diploid cells. *Antimicrob. Ag. Chemother.* **2**: 476–484.

HAVELL, E. A. and VILCEK, J. (1975) Inhibition of interferon secretion by vinblastine. *J. Cell Biol.* **64**: 716–719.

HAVELL, E. A., VILCEK, J., FALCOFF, E. and BERMAN, B. (1975b) Suppression of human interferon production by inhibitors of glycosylation. *Virology* **63**: 475–483.

HILL, D. A., BARON, S., LEVY, H. B., BELLANTI, J., BUCKLER, C. E., CANNELLOS, G., CARBONE, P., CHANOCK, R. M., DEVITA, V., GUGGENHEIM, M. A., HOMAN, E., KAPAKIAN, A. Z., KIRSCHSTEIN, R. L., MULLS, J., PERKINS, J. C., VAN KIRK, J. E. and WORTHINGTON, M. (1971) Clinical studies of induction of interferon by polyinosinic-polycytidylic acid. *Perspectives in Virology* **7**: 198–217.

HILL, D. A., BARON, S., PERKINS, J. C., WORTHINGTON, M., VAN KIRK, J. E., MILLS, J., KAPIKIAN, A. Z. and

CHANOCK, R. M. (1972) Evaluation of an interferon inducer in viral respiratory disease. *J. Am. med. Ass.* **219**: 1179–1184.

HILLER, G., JUNGWIRTH, C., BODO, G. and SCHULTZE, B. (1973) Biological activity of poly rI:rC: effect on poxvirus-specific functions. *Virology* **52**: 22–29.

HIRSCH, M. S., BLACK, P. H., WOOD, M. L. and MONACO, A. P. (1972) Effects of pyran copolymer on oncogenic virus infections in immunosuppressed hosts. *J. Immunol.* **108**: 1312–1318.

HO, M. (1964) Interferon-like viral inhibitor in rabbits after intravenous administration of endotoxin. *Science* **146**: 1472–1474.

HO, M. (1973) Factors influencing interferon production. In: *Interferons and Interferon Inducers*, pp. 73–105. FINTER, N. B. (ed.). American Elsevier, New York.

HO, M. and BREINIG, M. K. (1962) Conditions for the production of an interferon appearing in chick cell cultures infected with Sindbis Virus. *J. Immunol.* **89**: 177–186.

HO, M., BREINIG, M. C. and MAEHARA, N. (1976) Cellular basis of interferon formation and hyporeactivity after exposure to bacterial lipopolysaccharide. *J. Infect. Dis.* **133** (supplement): A30–A36.

HO, M., BREINIG, M. K., POSTIC, B. and ARMSTRONG, J. A. (1970) Effect of preinjections on the stimulation of interferon by a complexed polynucleotide, endotoxin and virus. *Ann. N.Y. Acad. Sci.* **173**: 680–693.

HO, M. and KE, Y. H. (1970) The mechanism of stimulation of interferon production by a complexed polyribonucleotide. *Virology* **40**: 693–702.

HO, M., KE, Y. H. and ARMSTRONG, J. A. (1973) Mechanism of interferon induction by endotoxin. *J. Infect. Dis.* **128** (supplement): S220–S227.

HO, M. and KONO, Y. (1965a) Tolerance to the induction of interferons by endotoxins and virus. *J. clin. Invest.* **44**, 1059–1060.

HO, M. and KONO, Y. (1965b) Effect of actinomycin D on virus and endotoxin-induced interferon-like inhibitors in rabbits. *Proc. natn. Acad. Sci. USA* **53**: 220–224.

HO, M., KONO, Y. and BREINIG, M. K. (1965) Tolerance of the induction of interferons by endotoxins and virus: role of a humoral factor. *Proc. Soc. exp. Biol. Med.* **119**: 1227–1232.

HO, M., TAN, Y. H. and ARMSTRONG, J. A. (1972) Accentuation of production of human interferon by metabolic inhibitors. *Proc. Soc. exp. Biol. Med.* **139**: 259–262.

HOFFMAN, W. W., KORST, J. J., NIBLACK, J. F. and CRONIN, T. H. (1973) N,N-dioctadecyl-N',N'-bis(2-hydroxyethyl)propanediamine: antiviral activity and interferon stimulation in mice. *Antimicrob. Ag. Chemother.* **3**: 498–502.

HOTERMAN, O. A. and HAVELL, E. A. (1970) Reduced interferon response in mice congenitally infected with lymphocytic choriomeningitis. *J. gen. Virol.* **9**: 101–103.

HOUCK, J. C. and BERMAN, L. B. (1958) Serum ribonuclease activity. *J. Appl. Physiol.* **12**: 473–476.

HOWARD, F. B., FRAZIER, J. and MILES, H. T. (1969) Interaction of poly-5-bromocytidylic acid with polyinosinic acid. *J. biol. Chem.* **244**: 1291–1301.

HUNT, T. and EHRENFELD, E. (1971) Cytoplasm from poliovirus-infected HeLa cells inhibits cell-free haemoglobin synthesis. *Nature* **230**: 91–94.

HUNTER, T., HUNT, T., JACKSON, R. J. and ROBERTSON, H. D. (1975) The characteristics of inhibiton of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. *J. biol. Chem.* **250**: 409–417.

HUPPERT, J., HILLOVA, J. and GRESLAND, L. (1969) Viral RNA synthesis in chicken cells infected with ultraviolet irradiated Newcastle disease virus. *Nature* **223**: 1015–1017.

HUTCHINSON, D. W., JOHNSTON, M. D. and EATON, M. A. W. (1974) Necessity for the 2'-hydroxyl group for the antiviral activity of synthetic polynucleotides. *J. gen. Virol.* **23**: 331–333.

HUTCHINSON, D. W. and MERIGAN, T. C. (1975) The lack of antiviral effect of (polyinosinic acid):(poly-cytidylic acid) when attached to insoluble supports. *J. gen. Virol.* **27**: 403–407.

ISAACS, A. and BURKE, D. C. (1958) Mode of action of interferon. *Nature* **182**: 1073–1074.

ISAACS, A. and LINDENMANN, J. (1957). Virus interference. I. The interferon. *Proc. R. Soc.* B **147**: 258–267.

ITO, Y., KUNII, A., MORI, N. and NAGATA, I. (1971) Effects of splenectomy on production of endotoxin-type interferon in mice. *Virology* **44**: 638–641.

ITO, Y., NAGATA, I. and KUNII, A. (1973) Mechanism of endotoxin-type interferon production in mice. *Virology* **52**: 439–446.

JAHIEL, R. I., TAYLOR, D., RAINFORD, N., HIRSCHBERG, S. E. and KROMAN, R. (1971) Inducers of interferon inhibit the mitotic response of liver cells to partial hepatectomy. *Proc. natn. Acad. Sci. USA* **68**: 740–742.

JAHIEL, R. I., VILCEK, J., NUSSENZWEIG, R. and VANDERBERG, J. (1968) Interferon inducers protect mice against *Plasmodium berghei* malaria. *Science* **161**: 802–804.

JANIK, B. (1971) *Physicochemical Characteristics of Oligonucleotides and Polynucleotides.* IFI/Plenum, New York.

JANKOWSKI, W. J., DAVEY, M. W., O'MALLEY, J. A., SULKOWSKI, E. and CARTER, W. A. (1975) Molecular structure of human fibroblast and leukocyte interferons: probe by lectin and hydrophobic chromatography. *J. Virol.* **16**: 1124–1130.

JAY, G., ABRAMS, W. R. and KAEMPFER, R. (1974) Resistance of bacterial protein synthesis to double-stranded RNA. *Biochem. biophys. Res. Commun.* **60**: 1357–1364.

JOHNSTON, M. D., ATHERTON, K. T., HUTCHINSON, D. W. and BURKE, D. C. (1976) The binding of poly(rI)-poly(rC) to human fibroblasts and the induction of interferon. *Biochim. biophys. Acta* **435**: 69–75.

JOHNSTON, M. I., STOLLAR, B. D., TORRENCE, P. F. and WITKOP, B. (1975) Structural features of double-stranded polyribonucleotides required for immunological specificity and interferon induction. *Proc. natn. Acad. Sci. USA* **72**: 4564–4568.

JOVIN, T. M. (1976) Recognition mechanisms of DNA-specific enzymes. *A. Rev. Biochem.* **45**: 889–920.

JULLIEN, P. and DE MAEYER-GUIGNARD, J. (1971) Toxic effect of polyriboinosinic-polyribocytidylic acid on mouse hemopoietic stem cells. *Int. J. Cancer* **7**: 468–475.

KAEMPFER, R. (1974) Identification and RNA-binding properties of an initiation factor capable of relieving translational inhibition induced by heme deprivation or double-stranded RNA. *Biochem. biophys. Res. Commun.* 61: 591–597.

KAEMPFER, R. and KAUFMAN, J. (1973) Inhibition of cellular protein synthesis by double-stranded RNA: inactivation of an initiation factor. *Proc. natn. Acad. Sci. USA* **70**: 1222–1226.

KALMAKOFF, J. and AUSTIN, F. J. (1973) Induction of viral interference: effects of poly rI·rC and diethylaminoethyldextran on the activity of the antiviral protein. *Infect. Immunity* **8**: 63–67.

KAMM, R. C. and SMITH, A. G. (1972) Ribonuclease activity in human plasma. *Clin. Biochem.* **5**: 198–200.

KAPILA, K., SMITH, C. and RUBIN, A. A. (1971) Effect of pyran copolymer on phagocytosis and tumor growth. *J. Reticul. Soc.* **9**: 447–450.

KAPLAN, A. M., MORAHAN, P. S. and REGELSON, W. (1974) Induction of macrophage-mediated tumor-cell cytotoxicity by pyran copolymer. *J. natn. Cancer Inst.* **52**: 1919–1921.

KATO, N., NAKASHIMA, I. and OHTA, M. (1975) Interferon production in mice by the capsular polysaccharide of *Klebsiella pneumoniae*. *Infect. Immunity* **12**: 1–6.

KATZ, E., MARGALITH, E. and WINER, B. (1976a) Inhibition of herpes virus deoxyribonucleic acid and protein synthesis by tilorone hydrochloride. *Antimicrob. Ag. Chemother.* **9**: 189–195.

KATZ, E., MARGALITH, E. and WINER, B. (1976b) The effect of tilorone hydrochloride on the growth of several animal viruses in tissue cultures. *J. gen. Virol.* **31**: 125–129.

KAUFMAN, H. E., CENTIFANTO, Y. M., ELLISON, E. D. and BROWN, D. C. (1971) Tilorone hydrochloride: human toxicity and interferon stimulation. *Proc. Soc. exp. Biol. Med.* **137**: 357–360.

KAWADE, Y. and UJIHARA, M. (1969) Non-inducing RNA antagonizes the induction of interferon with animal virus infection. *Nature* **221**: 569–570.

KELETI, G., FEINGOLD, D. S. and YOUNGNER, J. S. (1974) Interferon induction in mice by lipopolysaccharide from *Brucella abortus*. *Infect. Immunity* **10**: 282–283.

KERN, E. R., GLASGOW, L. A. and OVERALL, J. C., JR. (1976a) Antiviral activity of an extract of Brucella abortus: induction of interferon and immunopotentiation of host resistance. *Proc. Soc. exp. Biol. Med.* **152**: 372–376.

KERN, E. R., HAMILTON, J. R., OVERALL, J. C., JR. and GLASGOW, L. A. (1976b) Antiviral activity of BL-3849A, a low-molecular-weight oral interferon inducer. *Antimicrob. Ag. Chemother.* **10**: 691–696.

KERR, I. M., BROWN, R. E. and BALL, L. A. (1974) Increased sensitivity of cell-free protein synthesis to double-stranded RNA after interferon treatment. *Nature* **250**: 57–59.

KERR, I. M., BROWN, R. E., CLEMENS, M. J. and GILBERT, C. S. (1976) Interferon-mediated inhibition of cell-free protein synthesis in response to double-stranded RNA. *Eur. J. Biochem.* **69**: 551–561.

KIMBALL, P. C. and DUESBERG, P. H. (1971) Virus interference by cellular double-stranded ribonucleic acid. *J. Virol.* 7: 697–706.

KJELDSBERG, E. and FLIKKE, M. (1971) Antiviral activity of polyinosinic-polycytidylic acid in the absence of cell-controlled RNA synthesis. *J. gen. Virol.* **10**: 147–154.

KLEINSCHMIDT, W. J. (1972) Biochemistry of interferon and its inducers. *A. Rev. Biochem.* **41**: 517–542.

KLEINSCHMIDT, W. J., BOECK, L. V. D., VAN FRANK, R. M. and MURPHY, E. B. (1973) Interferon production by Ø6 pseudomonas phaseolicola phage and its double-stranded RNA. *Proc. Soc. exp. Biol. Med.* **144**: 304–307.

KLEINSCHMIDT, W. J., DOUTHART, R. J. and MURPHY, E. B. (1970) Interferon production by T4 coliphage. *Nature* **228**: 27–30.

KLEINSCHMIDT, W. J., ELLIS, L. F., VAN FRANK, R. M. and MURPHY, E. B. (1968) Interferon stimulation by a double-stranded RNA of a mycophage in statolon preparations. *Nature* **220**: 167–168.

KLEINSCHMIDT, W. J., VAN ETTEN, J. L. and VIDAVER, A. K. (1974) Influence of molecular weights of bacteriophage Ø6 double-stranded ribonucleic acids on interferon induction. *Infect. Immunity* **10**: 284–285.

KLIMPEL, G. R., DAY, K. D. and LUCAS, D. O. (1975) Differential production of interferon and lymphotoxin by human tonsil lymphocytes. *Cell. Immunol.* **20**: 187–196.

KNIGHT, E., JR. (1974a) Interferon-Sepharose: induction of the antiviral state. *Biochem. biophys. Res. Comm.* **56**: 860–864.

KNIGHT, E., JR. (1974b) Interferon-Sepharose: priming of L cells for induction of interferon by MM virus. *J. gen. Virol.* **25**: 143–145.

KOBAYASHI, S., YASUI, O. and MASUZUMI, M. (1969) Studies on early-appearing interferon *in vitro*. I. Production of endotoxin-induced interferon by mouse spleen cells cultured *in vitro*. *Proc. soc. exp. Biol. Med.* **131**: 487–494.

KOHASE, M. and VILČEK, J. (1977) Regulation of human interferon production stimulated with poly(I)·poly(C): correlation between shutoff and hyporesponsiveness to reinduction. *Virology* **76**: 47–54.

KOJIMA, Y., HOMMA, J. Y. and ABE, C. (1971) Property to induce interferon of *Pseudomonas aeruginosa* endotoxin and its components. *Jap. J. exp. Med.* **41**: 493–496.

KOJIMA, Y., YOSHIDA, F. and NAKASE, Y. (1973) Interferon production by *Bordetella pertussis* components in rabbits and in rabbit cell cultures. *Jap. J. Microbiol.* **17**: 160–161.

KOTAI, A., GANTI, T. and MECS, I. (1975) Polycationic modified polypeptides enhancing poly I:C induced viral resistance. *Experientia* **31**: 1165.

KRAMER, M. J., CLEELAND, R. and GRUNBERG, E. (1976) Antiviral activity of 10-carboxymethyl-9-acridanone. *Antimicrob. Ag. Chemother.* **9**: 233–238.

KRONENBERG, L. H. and FRIEDMANN, T. (1975) Relative quantitative assay of the biological activity of interferon messenger ribonucleic acid. *J. gen. Virol.* **27**: 225–238.

KRUEGER, R. F. and MAYER, G. D. (1970) Tilorone hydrochloride: an orally active antiviral agent. *Science* **169**: 1213–1214.

KRUEGER, R. F., MAYER, G. D., YOSHIMURA, S. and LUDWIG, K. A. (1971) *In vivo* evaluations of tilorone hydrochloride against Semliki Forest virus. *Antimicrob. Ag. Chemother.* **1970**: 486–490.

LACOUR, F., NAHON-MERLIN, E. and MICHELSON, M. (1973) Immunological recognition of polynucleotide

structure. In: *Current Topics in Microbiology and Immunology*. pp. 1–39, Vol. 62, BOSCHKE, F. D. (ed.). Springer-Verlag, New York.

LAI, M.-H. T. and JOKLIK, W. K. (1973) The induction of interferon by temperature-sensitive mutants of reovirus, UV-irradiated reovirus, and subviral reovirus particles. *Virology* **51**: 191–204.

LAMPSON, G. P., FIELD, A. K., TYTELL, A. A., NEMES, M. M. and HILLEMAN, M. R. (1970) Relationship of molecular size of $rI_n{:}rC_n$ (Poly I:C) to induction of interferon and host resistance. *Proc. Soc. exp. Biol. Med.* **135**: 911–916.

LAMPSON, G. P., NEMES, M. M., FIELD, A. K., TYTELL, A. A. and HILLEMAN, M. R. (1972) The effect of altering the size of poly C on the toxicity and antigenicity of poly I-C. *Proc. Soc. exp. Biol. Med.* **141**: 1068–1072.

LAMPSON, G. P., TYTELL, A. A., FIELD, A. K., NEMES, M. M. and HILLEMAN, M. R. (1967) Inducers of interferon and host resistance. I. Double-stranded RNA from extracts of *Penicillium funiculosum*. *Proc. natn. Acad. Sci.* **58**: 782–789.

LAMPSON, G. P., TYTELL, A. A., FIELD, A. K., NEMES, M. M. and HILLEMAN, M. R. (1969) Influence of polyamines on induction of interferon and resistance to viruses by synthetic polynucleotides. *Proc. Soc. exp. Biol. Med.* **132**: 212–218.

LEBLEU, B., SEN, G. C., SHAILA, S., CABRER, B. and LENGYEL, P. (1976) Interferon, double-stranded RNA, and protein phosphorylation. *Proc. natn. Acad. Sci.* **73**: 3107–3111.

LECHNER, M. C. and DUQUE MAGALHAES, M. C. (1973) RNase activities in blood serum of several vertebrates. *Experientia* **29**: 1479–1480.

LEUNEN, J., DESMYTER, J. and DE SOMER, P. (1971) Effects of oxyamylose and polyacrylic acid on foot-and-mouth disease and hog cholera virus infections. *Appl. Microbiol.* **21**: 203–208.

LEVINE, S., GIBSON, J. P. and MEGEL, H. (1974) Selective depletion of thymus dependent areas in lymphoid tissue by tilorone. *Proc. Soc. exp. Biol. Med.* **146**: 245–248.

LEVY, H. B., BAER, G., BARON, S., BUCKLER, C. E., GIBBS, C. J., IADAROLA, M. J., LONDON, W. T. and RICE, J. (1975) A modified polyriboinosinic-polyribocytidylic acid complex that induces interferon in primates. *J. Infect. Dis.* **132**: 434–439.

LEVY, H. B., BUCKLER, C. E. and BARON, S. (1966) Effect of interferon on early interferon production. *Science* **152**: 1274–1276.

LEVY, H. B., DUENWALD, J. and BUCKLER, C. E. (1973) Chlorite-oxidized amylose as an adjuvant for interferon production. *Infect. Immunity* **7**: 457–460.

LEVY, H. B., LONDON, W., PUCCILLO, D. A., BARON, S. and RICE, J. (1976) Prophylactic control of simian hemorrhagic fever in monkeys by an interferon inducer, polyriboinosinic-polyribocytidylic acid-poly-L-lysine. *J. Infect. Diseases* **133** (supplement): A256–A259.

LEVY, H. B. and RILEY, F. (1970) The effect of polyinosinic:polycytidylic acid on tumor metabolism. *Proc. Soc. exp. Biol. Med.* **135**: 141–145.

LEVY-KOENIG, R. E., GOLGHER, R. R. and PAUCKER, K. (1970) Immunology of interferons. II. Heterospecific activities of human interferons and their neutralization by antibody. *J. Immunol.* **104**: 791–797.

LIBONATI, M. (1971) Degradation of poly(A) and double-stranded RNA by aggregates of pancreatic ribonuclease. *Biochim. biophys. Acta* **228**: 440–445.

LINDAHL-MAGNUSSON, P., LEARY, P. and GRESSER, I. (1972) Interferon inhibits DNA synthesis induced in mouse lymphocyte suspensions by phytohaemagglutinin or by allogeneic cells. *Nature New Biol.* **237**: 120–121.

LINDNER-FRIMMEL, S. J. (1974) Enhanced production of human interferon by UV-irradiated cells. *J. gen. Virol.* **25**: 147–150.

LINDSAY, H. L., TROWN, P. W., BRANDT, J. and FORBES, M. (1969) Pyrogenicity of polyI·polyC in rabbits. *Nature* **223**: 717–718.

LO, G. and TREAGAN, L. (1974) Selective decrease in interferon production in immunotolerant mice. *Infect. Immunity* **9**: 192–194.

LOCKART, R. Z., JR. (1963) Production of an interferon by L cells infected with Western equine encephalomyelitis virus. *J. Bacteriol.* **85**: 556–566.

LOCKART, R. Z., JR., BAYLISS, N. L., TOY, S. T. and YIN, F. H. (1968) Viral events necessary for the induction of interferon in chick embryo cells. *J. Virol.* **2**: 962–965.

LOMANT, A. J. and FRESCO, J. R. (1975) Structural and energetic consequences of noncomplementary base oppositions in nucleic acid helices. *Prog. Nucleic Acid Res. Molec. Biol.* **15**: 185–218.

LOMNICZI, B. and BURKE, D. C. (1970) Interferon production by temperature-sensitive mutants of Semliki Forest virus. *J. gen. Virol.* **8**: 55–68.

LONG, W. F. and BURKE, D. C. (1971) Interferon production by double-stranded RNA: a comparison of induction by reovirus to that by a synthetic double-stranded polynucleotide. *J. gen. Virol.* **12**: 1–11.

LUKAS, B. and HRUSKOVA, J. (1968) *In vivo* induction of an interferon-like inhibitor by kanamycin. *Acta Virol.* **12**: 263–267.

MACHIDA, H., KUNINAKA, A. and YOSHINO, H. (1976) Relationship between the molecular size of polyI·polyC and its biological activity. *Jap. J. Microbiol.* **20**: 71–76.

MAHDY, M. S. and HO. M. (1964) Potentiating effect of fractions of Eastern equine encephalomyelitis virus on interferon production. *Proc. Soc. exp. Biol. Med.* **116**: 174–177.

MANGALIK, A., ROBINSON, W. A. and NATH, I. (1975) The effect of synthetic double-stranded polyribonucleotides on colony forming cells in normal human bone marrow. *Proc. Soc. exp. Biol. Med.* **148**: 816–818.

MARGOLIS, S. A., OIE, H. and LEVY, H. B. (1972) The effect of interferon, interferon inducers, or interferon induced virus resistance on subsequent interferon production. *J. gen. Virol.* **15**: 119–128.

MARKOVITS, P. and COPPEY, J. (1972) Studies on interferon induction by the avian adeno-virus CELO in chicken cells. *J. gen. Virol.* **17**: 157–162.

MARTELLY, I. and JULLIEN, P. (1974) Effect of repeated injections of polyriboinosinic-polyribocytidylic acid on mouse hematopoietic stem cells. *J. natn. Cancer Inst.* **53**: 1021–1025.

MATSUDA, S., KIDA, M., SHIRAFUJI, H., YONEDA, M. and YAOI, H. (1971) Induction of interferon and host resistance *in vivo* by double-stranded complexes of copolyribonucleotide of inosinic and guanylic acids with polyribocytidylic acid. *Arch. Ges. Virusforsch.* **34**: 105–118.

MAYER, G. D. and KRUEGER, R. F. (1970) Tilorone hydrochloride: mode of action. *Science* **169**: 1214–1215.

MAZUMDAR, S. K., SAENGER, W. and SCHEIT, K. H. (1974) Molecular structure of poly-2-thiouridylic acid, a double-helix with non-equivalent polynucleotide chains. *J. molec. Biol.* **85**: 213–229.

MCCORD, R. S., BREINIG, M. K. and MORAHAN, P. S. (1976) Antiviral effect of pyran against systemic infection of mice with herpes simplex virus type 2. *Antimicrob. Ag. Chemother.* **10**: 28–33.

MCNEILL, T. A. (1971) The effect of synthetic double-stranded polyribonucleotides on haemopoietic colony-forming cells *in vitro*. *Immunology* **21**: 741–750.

MEAGER, A. and BURKE, D. C. (1972) Production of interferon by ultraviolet radiation inactivated Newcastle disease virus. *Nature* **235**: 280–282.

MECS, I. and ROSZTOCZY, I. (1971) Physical and biological properties of DEAE-dextran polyriboinosinic:polyribocytidylic acid complexes. *Acta Virol.* **15**: 280–285.

MEGEL, H., RAYCHAUDHURI, A., GOLDSTEIN, S., KINSOLVING, C. R., SHEMANO, I. and Michael, J. G. (1974) Tilorone: its selective effects on humoral and cell-mediated immunity. *Proc. Soc. exp. Biol. Med.* **145**: 513–518.

MEGEL, H., RAYCHAUDHURI, A., SHEMANO, I., BEAVER, T. H. and THOMAS, L. L. (1975) The anti-inflammatory actions of tilorone hydrochloride. *Proc. Soc. Exp. Biol. Med.* **149**: 89–93.

MEHRISHI, J. N. (1970) Cell surface charge increase by both tumor inhibitory and tumor growth promoting polyanions. *Nature* **228**: 364–365.

MEHRISHI, J. N. (1972) Molecular aspects of the mammalian cell surface. *Prog. Biophys. molec. Biol.* **25**: 1–69.

MERIGAN, T. C. (1967) Induction of circulating interferon by synthetic anionic polymers of known composition. *Nature* **214**: 416–417.

MERIGAN, T. C. (1973) Non-viral substances which induce interferon. In: *Interferons and Interferon Inducers*, pp. 45–72, FINTER, N. B. (ed.). American Elsevier, New York.

MERIGAN, T. C. and FINKELSTEIN, M. S. (1968) Interferon-stimulating and *in vivo* antiviral effects of various synthetic anionic polymers. *Virology* **35**: 363–374.

MERIGAN, T. C. and REGELSON, W. (1967) Interferon induction in man by a synthetic polyanion of defined composition. *New Engl. J. Med.* **277**: 1283–1287.

MERIGAN, T. C. and ROTTMAN, F. (1974) Influence of increasing 2'-O-methylation on the interferon stimulating capacity of poly(rI)·poly(rC). *Virology* **60**: 297–301.

MICHELSON, A. M., MASSOULIE, J. and GUSCHLBAUER, W. (1967) Synthetic polynucleotides. *Prog. Nucl. Acid Res. molec. Biol.* **6**: 83–141.

MICHELSON, A. M., MONNY, C., LAURSEN, R. A. and LEONARD, N. J. (1966) Polynucleotide analogs. VIII. Poly 3-isoadenylic acid. *Biochim. biophys. Acta* **119**: 258–267.

MILES, H. T. and FRAZIER, J. (1964) A strand disproportionation reaction in a helical polynucleotide system. *Biochem. biophys. Res. Comm.* **14**: 21–28.

MILLER, J. H. and SOBELL, H. M. (1966) A molecular model for gene repression. *Proc. natn. Acad. Sci. USA* **55**: 1201–1205.

MILSTONE, L. M. and WAKSMAN, B. H. (1970) Release of virus inhibitor from tuberculin-sensitized peritoneal cells stimulated by antigen. *J. Immunol.* **105**: 1068–1071.

MOBRAATEN, L. E., DE MAEYER, E. and DE MAEYER-GUIGNARD, J. (1973) Prolongation of allograft survival in mice by inducers of interferon. *Transplantation* **16**: 415–420.

MOHR, S. J., BROWN, D. G. and COFFEY, D. S. (1972) Size requirement of polyinosinic acid for DNA synthesis, viral resistance and increased survival of leukaemic mice. *Nature New Biol.* **240**: 250–252.

MOHR, S. J., CHIRIGOS, M. A., FUHRMAN, F. and SMITH, G. (1976) Enhancement of a tumor allograft in BALB/c×DBA/2F, mice by pyran copolymer. *Cancer Res.* **36**: 1315–1319.

MONTAGNIER, L., COLLAVORE, H., DE MAEYER-GUIGNARD, J. and DE MAEYER, E. (1974) Two forms of mouse interferon messenger RNA. *Biochem. biophys. Res. Commun.* **59**: 1031–1038.

MORAHAN, P. S. and GROSSBERG, S. E. (1970) Age-related cellular resistance of the chicken embryo to viral infections III. *Escherichia coli* resistance-inducing protein. *Proc. Soc. exp. Biol. Med.* **134**: 342–347.

MORAHAN, P. S. and MCCORD, R. S. (1975) Resistance to herpes simplex type 2 virus induced by an immunopotentiator (pyran) in immunosuppressed mice. *J. Immunol.* **115**: 311–313.

MORAHAN, P. S., MUNSON, A. E., REGELSON, W., COMMERFORD, S. L. and HAMILTON, L. D. (1972a) Antiviral activity and side effects of polyriboinosinic-cytidylic acid complexes as affected by molecular size. *Proc. natn. Acad. Sci. USA* **69**: 842–846.

MORAHAN, P. S., REGELSON, W. and MUNSON, A. E. (1972b) Pyran and polyribonucleotides: differences in biological activities. *Antimicrob. Ag. Chemother.* **2**: 16–22.

MOZES, L. W., HAVELL, E. A., GRADOVILLE, M. L. and VILCEK, J. (1974) Increased interferon production in human cells irradiated with ultraviolet light. *Infect. Immunity* **10**: 1189–1191.

MOZES, L. W. and VILCEK, J. (1974) Interferon induction in rabbit cells irradiated with UV light. *J. Virol.* **13**: 646–651.

MOZES, L. W. and VILCEK, J. (1975) Distinguishing characteristics of interferon induction with poly(I)·poly(C) and Newcastle disease virus in human cells. *Virology* **65**: 100–111.

MUNSON, A. E., MUNSON, J. A., REGELSON, W. and WAMPLER, G. L. (1972) Effect of tilorone hydrochloride and congeners on reticuloendothelial system, tumors, and the immune response. *Cancer Res.* **32**: 1397–1403.

MUNSON, A. E., REGELSON, W., LAWRENCE, W., JR. and WOOLES, W. R. (1970) Biphasic response of the reticuloendothelial system (RES) induced by pyran copolymer. *J. Reticul. Soc.* **7**: 375–385.

MYERS, M. W. and FRIEDMAN, R. M. (1971) Potentiation of human interferon production by superinduction, *J. natn. Cancer Inst.* **47**: 757–764.

NEMES, M. M., TYTELL, A. A., LAMPSON, G. P., FIELD, A. K. and HILLEMAN, M. R. (1969) Inducers of interferon and host resistance VII. Antiviral efficacy of double-stranded RNA of natural origin. *Proc. Soc. exp. Biol. Med.* **132**: 784–789.

NG, M. H., BERMAN, B. and VILCEK, J. (1972) Membrane-bound intracellular interferon in rabbit kidney cell cultures. *Virology* **49**: 322–325.

NG, M. H. and VILCEK, J. (1972) Interferons: physiochemical properties and control of cellular synthesis. *Adv. Protein Chem.* **26**: 173–241.

NG, M. H. and VILCEK, J. (1973) Temperature-sensitive interferon production in rabbit kidney cell cultures treated with toyocamycin. *Biochim. biophys. Acta* **294**: 284–291.

NIBLACK, J. F. and MCCHEARY, M. B. (1971) Relationship of biological activities of poly(I)-poly(C) to homopolymer molecular weights. *Nature New Biol.* **233**: 52–53.

NICHOL, F. R., WEED, S. D. and UNDERWOOD, G. E. (1976) Stimulation of murine interferon by a substituted pyrimidine. *Antimicrob. Ag. Chemother.* **9**: 433–439.

NORDLUND, J. J., WOLFF, S. M. and LEVY, H. B. (1970) Inhibition of biologic activity of poly I: poly C by human plasma. *Proc. Soc. exp. Biol. Med.* **133**: 439–444.

NOVOKHATSKY, A. S., ERSHOV, F. I., TIMKOVSKY, A. L., BRESLER, S. E., KOGAN, E. M. and TIKHOMIROVA-STOROVA, N. S. (1975) Double-stranded complex of polyguanylic and polycytidylic acids and its antiviral activity in tissue culture. *Acta Virol.* **19**: 121–129.

O'MALLEY, J. A., HO, Y. K., CHAKRABARTI, P., DIBERARDINO, L., CHANDRA, P., ORINDA, D. A. O., BYRD, D. M., BARDOS, T. J. and CARTER, W. A. (1975) Antiviral activity of partially thiolated polynucleotides. *Mol. Pharmac.* **11**: 61–69.

OSBURN, J. E. and MEDAERIS, D. N., JR. (1967) Suppression of interferon and antibody and multiplication of Newcastle disease virus in cytomegalovirus infected mice. *Proc. Soc. exp. Biol. Med.* **124**: 347–355.

PANUSARN, C., STANLEY, E. D., DIEDA, V., RUBENIS, M. and JACKSON, G. G. (1974) Prevention of illness from rhinovirus infection by a topical interferon inducer. *New Engl. J. Med.* **291**: 57–61.

PAPAS, T. S., PRY, T. W. and CHIRIGOS, M. A. (1974) Inhibition of RNA-dependent DNA polymerase of avian myeloblastosis virus by pyran copolymer. *Proc. natn. Acad. Sci. USA* **71**: 367–370.

PATHAK, P. N. and TOMPKINS, W. A. F. (1974) Interferon production by macrophages from adult and newborn rabbits bearing fibroma virus-induced tumors. *Infect. Immunity* **9**: 669–673.

PAUCKER, K. and BOXACA, M. (1967) Cellular resistance to induction of interferon. *Bacterial. Rev.* **31**: 145–156.

PESTKA, S., MCINNES, J., HAVELL, E. A. and VILCEK, J. (1975) Cell-free synthesis of human interferon. *Proc. natn. Acad. Sci. USA* **72**: 3898–3901.

PETTIJOHN, D. E. and HECHT, R. (1973) RNA molecules bound to the folded bacterial genome stabilize DNA folds and segregate domains of supercoiling. *Cold Spring Harbor Sym. Quant. Biol.* **38**: 31–41.

PINDAK, F. F. (1970) Protection of mice against bacterial infection by interferon inducers. *Infect. Immunity* **1**: 271–273.

PINDAK, F. F., SCHMIDT, J. P., GIRON, D. J. and ALLEN, P. T. (1971) Interferon levels and resistance to viral infection associated with selected interferon inducers. *Proc. Soc. exp. Biol. Med.* **138**: 317–321.

PITHA, P. M. and CARTER, W. A. (1971a) Antiviral activity produced by the polycytidylic acid-hexainosinate system. *Nature New Biol.* **234**: 105–106.

PITHA, P. M. and CARTER, W. A. (1971b) The DEAE-dextran: polyriboinosinate-polyribocytidylate complex: physical properties and interferon induction. *Virology.* **45**: 777–781.

PITHA, P. M., HARPER, H. D. and PITHA, J. (1974) Dependence of interferon induction on cell membrane integrity. *Virology* **59**: 40–50.

PITHA, P. M. and HUTCHINSON, D. W. (1977) The mechanism of interferon induction by synthetic polynucleotides. (in press).

PITHA, P. M., MARSHALL, L. W. and CARTER, W. A. (1972) Interferon induction: rate of cellular attachment of poly IC. *J. gen. Virol.* **15**: 89–92.

PITHA, J. and PITHA, P. M. (1971) Antiviral resistance by the polyinosinic acid–poly(1-vinylcytosine) complex. *Science* **172**: 1146–1148.

PITHA, P. M. and PITHA, J. (1973) Interferon induction site: poly IC on solid substrate carriers. *J. gen. Virol.* **21**: 31–37.

PITHA, P. M. and PITHA, J. (1974) Interferon induction by single-stranded polynucleotides modified with polybases. *J. gen. Virol.* **24**: 385–390.

PLANTEROSE, D. N., BIRCH, P. J., PILCH, D. J. F. and SHARPE, T. J. (1970) Antiviral activity of double stranded RNA and virus-like particles from *Penicillium stoloniferum*. *Nature* **227**: 594–505.

POCHON, F. and MICHELSON, A. M. (1965) Polynucleotides. VI. Interaction between polyguanylic acid and polycytidylic acid. *Proc. natn. Acad. Sci. USA* **53**: 1425–1430.

PRUSINER, P., BRENNAN, T. and SUNDARALINGAM, M. (1973) Crystal structure and molecular conformation of formycin monohydrates. Possible origin of the anamolous circular dichroic spectra in formycin mono- and polynucleotides. *Biochemistry* **12**: 1196–1202.

PURCELL, R. H., GERIN, J. L., LONDON, W. T., WAGNER, J. A., MCAULIFFE, V. J., POPPER, H., PALMER, A. E., LVOVSKY, E., KAPLAN, P. M., WONG, D. C. and LEVY, H. B. (1976) Modifications of chronic hepatitis-B virus infection in chimpanzees by administration of an interferon inducer. *Lancet* **ii**: 757–761.

PUSZTAI, R., BELADI, I., BAKAY, M. and MUCSI, I. (1969) Effect of ultraviolet irradiation and heating on the interferon-inducing capacity of human adenoviruses. *J. gen. Virol.* **4**: 169–176.

RAINEN, L. C. and STOLLAR, B. D. (1977) Antisera to poly(A)-poly(U)-poly(I) contain antibody subpopulations specific for different aspects of the triple-helix. *Biochemistry* **16**: 2003–2007.

RASMUSSEN, L. E., JORDAN, G. W., STEVENS, D. A. and MERIGAN, R. C. (1974) Lymphocyte interferon production and transformation after herpes simplex infections in humans. *J. Immunol.* **112**: 728–736.

RAYCHAUDHURI, A. and MEGEL, H. (1976) The effect of tilorone on immunoglobulin-bearing lymphocytes (B cells) in peripheral blood and spleens of mice. *J. Reticul. Soc.* **20**: 127–134.

REDDI, K. K. and HOLLAND, J. F. (1976) Elevated serum ribonuclease in patients with pancreatic cancer. *Proc. natn. Acad. Sci. USA* **73**: 2308–2310.

REIJNDERS, L., AALBERS, A. M. J., VAN KAMMEN, A. and BERNS, A. J. M. (1975) The effect of double-stranded cowpea mosaic viral RNA on protein synthesis. *Biochim. biophys. Acta* **390**: 69–77.

REMINGTON, J. S. and MERIGAN, T. C. (1970) Synthetic polyanions protect mice against intracellular bacterial infection. *Nature* **226**: 361–363.

REUSS, K., SCHEIT, K. -H. and SAIKO, O. (1976) Induction of interferon by polyribonucleotides containing thiopyrimidines. *Nucleic Acids Res.* **3**: 2861–2875.

REYNOLDS, F. H., JR. and PITHA, P. M. (1974) The induction of interferon and its messenger RNA in human fibroblasts. *Biochem. biophys. Res. Commun.* **59**: 1023–1030.

REYNOLDS, F. H., JR., PREMKUMAR, E. and PITHA, P. M. (1975) Interferon activity produced by translation of human interferon messenger RNA in cell-free ribosomal systems and in Xenopus oöcytes. *Proc. natn. Acad. Sci. USA* **72**: 4881–4885.

RICE, J. M., TURNER, W. and CHIRIGOS, M. A. (1970) Enhancement by poly-D-lysine of poly I:C-induced interferon production in mice. *Appl. Microbiol.* **19**: 867–869.

RICE, J. M., TURNER, W., CHIRIGOS, M. A. and SPAHN, G. (1971) Dose responsiveness and variation among inbred strains of mice in production of interferon after treatment with poly(I)-poly(C) [poly-D-lysine] complexes. *Appl. Microbiol.* **22**: 380–386.

RICHMOND, J. Y. (1969) An interferon-like inhibitor of foot-and-mouth disease virus induced by phytohemagglutinin in swine leukocyte cultures. *Arch. Ges. Virusforsch.* **27**: 282–289.

RICHMOND, J. Y. (1971a) Mouse resistance against foot-and-mouth disease virus induced by injections of pyran. *Infect. Immunity* **3**: 249–253.

RICHMOND, J. Y. (1971b) Diethylaminoethyl-dextran. Requirement for poly I:C-induced interferon effective against foot-and-mouth disease virus *in vitro*. *Arch. Ges. Virusforsch.* **33**: 242–250.

RICHMOND, J. Y. and CAMPBELL, C. H. (1972) Influence of divinyl ether-maleic anhydride (pyran) on foot-and-mouth disease virus infection: effect on adsorption and multiplication in mouse tissues. *Arch. Ges. Virusforsch.* **36**: 232–239.

RICHMOND, J. Y. and CAMPBELL, C. H. (1973) Foot-and-mouth disease virus: protection induced in infected mice by two orally administered interferon inducers. *Arch. Ges. Virusforsch.* **42**: 102–105.

RICHMOND, T. J. and STEITZ, T. A. (1976) Protein-DNA interaction investigated by binding *Escherichia coli lac* repressor protein to poly[d(A-U–HgX)]. *J. molec. Biol.* **103**: 25–38.

RINALDO, C. R., JR., COLE, B. C., OVERALL, J. C., JR. and GLASGOW, L. A. (1974) Induction of interferon in mice by mycoplasmas. *Infect. Immunity* **10**: 1296–1301.

RINALDO, C. R., JR., OVERALL, J. C., JR., COLE, B. C. and GLASGOW, L. A. (1973) Mycoplasma-associated induction of interferon in ovine leukocytes. *Infect. Immunity* **8**: 796–803.

ROBERTS, W. K., CLEMENS, M. J. and KERR, I. M. (1976a) Interferon-induced inhibition of protein synthesis in L-cell extracts: an ATP- dependent step in the activation of an inhibitor by double-stranded RNA. *Proc. natn. Acad. Sci. USA* **73**: 3136–3140.

ROBERTS, W. K., HOVANESSIAN, A., BROWN, R. E., CLEMENS, M. J. and KERR, I. M. (1976b) Interferon-mediated protein kinase and low-molecular-weight inhibitor of protein synthesis. *Nature* **264**: 477–480.

ROBERTSON, H. D. and MATHEWS, M. B. (1973) Double-stranded RNA as an inhibitor of protein synthesis and as a substrate for a nuclease in extracts of Krebs II ascites cells. *Proc. natn. Acad. Sci. USA* **70**: 225–229.

ROBERTSON, H. D., WEBSTER, R. E. and ZINDER, N. D. (1968) Purification and properties of ribonuclease III from *Escherichia coli*. *J. biol. Chem.* **243**: 82–91.

RODGERS, R. and MERIGAN, T. C. (1972) Interferon and its inducers: antiviral and other effects. *Crit. Rev. Clin. Lab. Sci.* **3**: 131–162.

ROSZTOCZY, I. (1971) Interferon induction by polyinosinic-polycytidylic acid in interferon pretreated L cells in absence of DEAE-dextran. *Acta Virol.* **15**: 431.

ROSZTOCZY, I. (1974) Further evidence that priming is a non-antiviral function of interferon. *Acta Microbiol. Acad. Sci. Hung.* **21**: 305–309.

ROSZTOCZY, I. and MECS, I. (1970) Enhancement of interferon synthesis by polyinosinic-polycytidylic acid in L cells pretreated with interferon. *Acta Virol.* **14**: 398–400.

ROUSSET, S. (1974) Refractory state of cells to interferon induction. *J. gen. Virol.* **22**: 9–20.

ROZEE, K. R., LEE, S. H. S. and NGAN, J. (1973) Effect of priming on interferon inhibition of Con A induced spleen cell blastogenesis. *Nature New Biol.* **245**: 16–18.

RUHL, H., VOGT, W., ROCHERT, G. and DIAMANTSTEIN, T. (1974) Stimulation of B cells by poly A:poly U and poly I:poly C *in vitro*. *Immunology* **26**: 937–941.

SAITO, N., SUZUKI, F., SASAKI, K. and ISHIDA, N. (1976) Antiviral and interferon-inducing activity of a new glutarimide antibiotic, 9-methylstreptimidone. *Antimicrob. Ag. Chemother.* **10**: 14–19.

SALVIN, S. B., NISHIO, J. and SHONNARD, J. T. (1974) Two new inhibitory activities in blood of mice with delayed hypersensitivity, after challenge with specific antigen. *Infect. Immunity* **9**: 621–635.

SALVIN, S. B., RIHI, E., GRANGER, D. L. and YOUNGNER, J. S. (1975) Migration inhibitory factor and Type II interferon in the circulation of mice sensitized with mycobacterial components. *J. Immunol.* **114**: 354–359.

SALVIN, S. B., YOUNGNER, J. S. and LEDERER, W. H. (1973) Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. *Infect. Immunity* **7**: 68–75.

SCHAFER, M. P., CHIRIGOS, M. A. and PAPAS, T. S. (1974) Inhibition of Rauscher leukemia virus and avian myeloblastosis virus DNA polymerases by tilorone (NSC-143969) and its analog. *Cancer Chemother. Rep.* **58**: 821–827.

SCHAFER, T. W. and LOCKART, R. Z., JR. (1970) Interferon required for viral resistance induced by polyI·polyC. *Nature* **226**: 449–450.

SCHULTZ, R. M., PAPAMATHEAKIS, J. D., STYLOS, W. A. and CHIRIGOS, M. A. (1976) Augmentation of specific macrophage-mediated cytotoxicity: correlation with agents which enhance antitumor resistance. *Cell. Immunol.* **25**: 309–316.

SEHGAL, P. B., TAMM, I. and VILCEK, J. (1975a) Human interferon production: superinduction by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. *Science* **190**: 282–284.

SEHGAL, P. B., TAMM, I. and VILCEK, J. (1975b) Enhancement of human interferon production by neutral

red and chloroquine: analysis of inhibition of protein degradation and macromolecular synthesis. *J. exp. Med.* 142: 1283–1300.

SEHGAL, P. B. and TAMM, I. (1976) An evaluation of messenger RNA competition in the shut-off of human interferon production. *Proc. natn. Acad. Sci. USA* **73**: 1621–1625.

SEHGAL, P. B., TAMM, I. and VILCEK, J. (1976a) On the mechanism of enhancement of human interferon production by actinomycin D and cycloheximide. *Virology* **70**: 256–259.

SEHGAL, P. B., TAMM, I. and VILCEK, J. (1976b) Regulation of human interferon production. I. Superinduction by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. *Virology* **70**: 532–541.

SEHGAL, P. B., TAMM, I. and VILCEK, J. (1976c) Regulation of human interferon production. II. Inhibition of interferon messenger RNA synthesis by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. *Virology* **70**: 542–544.

SEN, G. C., LEBLEU, B., BROWN, G. E., KAWAKITA, M., SLATTERY, E. and LENGYEL, P. (1976) Interferon, double-stranded RNA and mRNA degradation. *Nature* **264**: 370–373.

SEROTA, F. T. and BASERGA, R. (1970) Polyinosinic acid-polycytidylic acid: inhibition of DNA synthesis stimulated by isoproterenol. *Science* **167**: 1379–1380.

SHARPE, T. J., BORCH, P. J. and PLANTEROSE, D. S. (1971) Resistance to virus infection during the hyporeactive state of interferon induction. *J. gen. Virol.* **12**: 331–333.

SCHEID, B., CHIN, A. E. and NELSON, J. H., JR. (1972) Serum-induced inhibition of enzymes which synthesize RNA or utilize RNA as a substrate. *Clin. Chim. Acta* **39**: 477–479.

SHENK, T. E. and STOLLAR, V. (1972) Effect of double-stranded RNA's from Sindbis virus-infected cells on rabbit reticulocyte and chick embryo cell-free synthesis. *Biochim. biophys. Acta* **287**: 501–513.

SHIOKAWA, L. and YAOI, H. (1972) Effects of sonication on physicochemical and biological properties of synthetic double-stranded polyribonucleotides as interferon inducer. *Arch. Ges. Virusforsch.* **38**: 109–124.

SHUGAR, D. and SIERAKOWSKA, H. (1967) Mammalian nucleotytic enzymes and their localization. *Prog. Nucl. Acid Res. Mol. Biol.* **7**: 369–429.

SIGLER, R. B., DAVIES, D. R. and MILES, H. T. (1962) A displacement reaction between a polynucleotide helix and a random coil. *J. molec. Biol.* **5**: 509–517.

SILL, A. D., ALBRECHT, W. L., ANDREWS, E. R., FLEMING, R. W., HORGAN, S. W., ROBERTS, E. MCC. and SWEET, F. W. (1973) Bis-basic-substituted polycyclic aromatic compounds. A new class of antiviral agents. 1. Bisalkamine esters of fluorenone-, fluorenol-, and fluorenedicarboxylic acids. *J. Med. Chem.* **16**: 240–245.

SILL, A. D., ANDREWS, E. R., SWEET, F. W., HOFFMAN, J. W., TIERNAN, P. L., GRISAR, J. M., FLEMING, R. W. and MAYER, G. D. (1974) Bis-basic-substituted polycyclic aromatic compounds. A new class of antiviral agents. 5. Bisbasic ethers of anthraquinone and bisalkamine esters of anthraquinone-dicarboxylic acids. *J. Med. Chem.* **17**: 965–968.

SIMHA, R. and ZIMMERMAN, J. M. (1960) Synthesis kinetics and sequence distribution in synthetic polynucleotides. *J. Polymer Sci.* **42**: 309–325.

SIMINOFF, P. (1975) *In vivo* and *in vitro* stimulation of mouse spleen leukocytes by BL-20803, a low-molecular-weight interferon inducer. *Infect. Immunity* **12**: 1051–1057.

SIMINOFF, P. (1976) Target cells for interferon induction by BL-20803. *J. Infect. Dis.* (supplement) **133**: 37–42.

SIMINOFF, P., BERNARD, A. M., HURSKY, V. S. and PRICE, K. E. (1973) BL-20803, a new, low-molecular-weight interferon inducer. *Antimicrob. Ag. Chemother.* **3**: 742–743.

SOEHNER, R. L., GAMBARDELLA, M. M. and HOU, E. F. (1974) Oral induction of interferon by a low-molecular-weight compound. *Proc. Soc. exp. Biol. Med.* **145**: 1114–1119.

SOLOVIEV, V. D., MARCHENKO, V. I., DYUISALIEVA, R. G., BABAYANTS, A. A. and DOKIDYSHEVA, L. N. (1970) Stimulation of interferogenesis in producing cells by pretreatment with interferon. *Vop. Virusol.* **15**: 150–155.

SOULELIL, C. and PANUEL, J. (1968) Immunochemistry of polyribonucleotides. Study of polyriboinosinic and polyriboguanylic acids. *Biochemistry* **7**: 7–13.

STERN, R. (1970) A nuclease from animal serum which hydrolyzes double-stranded RNA. *Biochem. biophys. Res. Comm.* **41**: 608–614.

STEVENS, G. and FELSENFELD, G. (1964) The conversion of two-stranded poly(A + U) to three-stranded poly(A + 2U) and poly(A) by heat. *Biopolymers* **2**: 293–314.

STEWARD, D. L., HERNDON, W. C., JR. and SCHELL, K. R. (1972) Influence of 2′-*O*-acetylation on the antiviral activity of polyribonucleotides. *Biochim. biophys. Acta* **262**: 227–232.

STEWART, W. E., II and DE CLERCQ, E. (1974) Relationship of cytotoxicity and interferon-inducing activity of polyriboinosinic acid-polyribocytidylic acid to the molecular weights of the homopolymers. *J. gen. Virol.* **23**: 83–89.

STEWART, W. E., II, DE CLERCQ, E., BILLIAU, A., DESMYTER, J. and DE SOMER, P. (1972a) Increased susceptibility of cells treated with interferon to the toxicity of polyriboinosinic-polyribocytidylic acid. *Proc. natn. Acad. Sci. USA* **69**: 1851–1854.

STEWART, W. E., II, DE CLERCQ, E. and DE SOMER, P. (1973a) Specificity of interferon-induced enhancement of toxicity for double-stranded ribonucleic acids. *J. gen. Virol.* **18**: 237–246.

STEWART, W. E., II, DE CLERCQ, E., DE SOMER, P., BERG, K., OGBURN, C. A. and PAUCKER, K. (1973b) Antiviral and non-antiviral activity of highly purified interferon. *Nature New Biol.* **246**: 141–143.

STEWART, W. E., II, GOSSER, L. B. and LOCKART, R. Z., JR. (1971a) Distinguishing characteristics of the interferon response of primary and continuous mouse cell cultures. *J. gen. Virol.* **13**: 35–50.

STEWART, W. E., II, GOSSER, L. B. and LOCKART, R. Z. (1971b) Priming: a nonantiviral function of interferon. *J. Virol.* **7**: 792–801.

STEWART, W. E., II, GOSSER, L. B. and LOCKART, R. Z., JR. (1972b) The effect of priming with interferon on interferon production by two lines of L cells. *J. gen. Virol.* **15**: 85–87.

STEWART, W. E., II, SCOTT, W. D. and SULKIN, S. E. (1969) Relative sensitivities of viruses of different species of interferon. *J. Virol.* **4**: 147–153.

STINEBRING, W. R. and ABSHER, P. M. (1970) Production of interferon following immune response. *Ann. N.Y. Acad. Sci.* **173**: 714–718.

STINEBRING, W. R. and YOUNGNER, J. S. (1964) Patterns of interferon appearance in mice injected with bacteria or bacterial endotoxin. *Nature* **204**: 712.

STOBO, J., GREEN, I., JACKSON, L. and BARON, S. (1974) Identification of a subpopulation of mouse lymphoid cells required for interferon production after stimulation with mitogens. *J. Immunol.* **112**: 1589–1593.

STOLLAR, B. D. (1973) Nucleic acid antigens. In: *The Antigens*, pp. 1–85, Vol. 1, SELA, M. (ed.). Academic Press, New York.

STOLLAR, B. D. (1975) The specificity and applications of antibodies to helical nucleic acids. *Crit. Rev. Biochem.* **3**: 45–69.

STRINGFELLOW, D. A. (1975) Hyporeactivity to interferon induction: characterization of a hyporeactive factor in the serum of encephalomyocarditis virus-infected mice. *Infect. Immunity* **11**: 294–302.

STRINGFELLOW, D. A. and GLASGOW, L. A. (1972a) Hyporeactivity of infection: potential limitation to therapeutic use of interferon-inducing agents. *Infect. Immunity* **6**: 743–747.

STRINGFELLOW, D. A. and GLASGOW, L. A. (1972b) Tilorone hydrochloride: an oral interferon-inducing agent. *Antimicrob. Ag. Chemother.* **2**: 73–78.

STRINGFELLOW, D. A. and GLASGOW, L. A. (1974) Hyporeactivity due to infection: recognition of a transferable hyporeactive factor in the serum of encephalomyocarditis virus-infected mice. *Infect. Immunity* **10**: 1337–1342.

STRINGFELLOW, D. A., OVERALL, J. C., JR. and GLASGOW, L. A. (1974) Interferon inducers in therapy of infection with encephalomyocarditis virus in mice. I. Effect of single doses of polyriboinosinic-polyribocytidylic acid and tilorone hydrochloride on viral pathogenesis. *J. Infect. Dis.* **130**: 470–479.

STUDDERT, D. S. and DAVIS, R. C. (1974) Calculations of double-helical nucleic acids. I. Effects involving $\pi \rightarrow \pi^*$ transitions. *Biopolymers*. **13**: 1377–1389.

SUBBOTINA, M. F., TIMKOVSKY, A. L., CHERNAENKO, V. M., BRESLER, S. E. and TIKHOMIROVA-SIDOROVA, N. S. (1972) Interaction of polyriboguanylic acid and polyribocytidylic acids. *Molek. Biol.* **6**: 817–823.

SUHADOLNIK, R. J. (1970) *Nucleoside Antibiotics*, p. 338. Wiley Interscience, New York.

SUNDARALINGAM, M. (1975) Principles governing nucleic acid and polynucleotide conformations. In: *Structure and Conformation of Nucleic Acids and Protein–Nucleic Acid Interactions*, pp. 487–524, SUNDARALINGAM, M. and RAO, S. T., (eds.). University Park Press, Baltimore.

SUZUKI, F., ISHIDA, N., SUZUKI, M., SATO, T. and SUZUKI, S. (1975) Effect of the interferon inducer, dextran phosphate, on influenza virus infection in mice. *Proc. Soc. exp. Biol. Med.* **149**: 1069–1075.

TABORSKY, I., DOSKOCIL, J. and ZAJICOVA, V. (1974) Pyrogenic activity of double-stranded RNA from *Escherichia coli* f_2 phage. *Acta. Virol.* **18**: 106–112.

TAKEHARA, M. and SUZUKI, N. (1973) Interferon induction by rice dwarf virus RNA. Antiviral activity *in vitro* and its stability. *Arch. Ges. Virusforsch.* **40**: 291–299.

TAN, Y. H. (1975) Chromosome-21 dosage effect on inducibility of antiviral gene(s). *Nature* **253**: 280–282.

TAN, Y. H., ARMSTRONG, J. A. and HO, M. (1971a) Intracellular interferon: kinetics of formation and release. *Virology* **45**: 837–840.

TAN, Y. H., ARMSTRONG, J. A. and HO, M. (1971b) Accentuation of interferon production by metabolic inhibitors and its dependence on protein synthesis. *Virology* **44**: 503–509.

TAN, Y. H., ARMSTRONG, J. A., KE, Y. H. and HO, M. (1970) Regulation of cellular interferon production: enhancement by antimetabolites. *Proc. natn. Acad. Sci. USA* **67**: 464–471.

TAN, Y. H., CREAGAN, R. P. and RUDDLE, F. H. (1974a) The somatic cell genetics of human interferon: assignment of human interferon loci to chromosomes 2 and 5. *Proc. natn. Acad. Sci. USA* **71**: 2251–2255.

TAN, Y. H., JENG, D. K. and HO, M. (1972) The release of interferon: an active process inhibited by *p*-hydroxymercuribenzoate. *Virology* **48**: 41–48.

TAN, Y. H., SCHNEIDER, E. L., TISCHFIELD, J., EPSTEIN, C. J. and RUDDLE, F. H. (1974b) Human chromosome-21 dosage: effect on the expression of the interferon induced antiviral state. *Science* **186**: 61–63.

TAN, Y. H., TISCHFIELD, J. and RUDDLE, F. H. (1973) The linkage of genes for the human interferon-induced antiviral protein and indophenol oxidase-B traits to chromosome G-21. *J. exp. Med.* **137**: 317–330.

TANFORD, C. (1961) *Physical Chemistry of Macromoles*, pp. 317–456. John Wiley, New York.

TAYLOR-PAPADIMITRIOU, J. and KALLOS, J. (1973) Induction of interferon by 'sepharose'-bound poly(I)·poly(C). *Nature New Biol.* **245**: 143–144.

TAZAWA, I., TAZAWA, S. and TSO, P. O. P. (1972) Studies on oligonucleotides: thermodynamic and optical properties of the oligoinosinate·polycytidylate complexes. *J. molec. Biol.* **66**: 115–130.

TENG, C. T., CHEN, M. C. and HAMILTON, L. D. (1973) poly(inosinic acid)·poly(cytidylic acid) inhibition of DNA synthesis in synchronized HeLa cells. *Proc. natn. Acad. Sci. USA* **70**: 3904–3908.

THACORE, H. R. and YOUNGNER, J. S. (1973) Different sensitivity of ribonucleic and deoxyribonucleic acid viruses to resistance induced in rabbit cells (RK-13) by polyriboinosinic acid·polyribocytidylic acid. *Infect. Immunity* **7**: 685–690.

THANG, M. N., BACHNER, L., DE CLERCQ, E. and STOLLAR, B. D. (1977) A continuous high molecular weight base-paired structure is not an absolute requirement for potential polynucleotide inducer of interferon. *FEBS Lett.* **76**: 159–165.

THANG, M. N., THANG, D. C., DE MAEYER, E. and MONTAGNIER, L. (1975) Biosynthesis of mouse interferon by translation of its messenger RNA in a cell-free system. *Proc. natn. Acad. Sci. USA* **72**: 3975–3977.

THIELE, D. and GUSCHLBAUER, W. (1973) The structures of polyinosinic acid. *Biophysik* **9**: 261–277.

TILLES, J. G. (1970) Diethylamino-dextran enhancement of interferon induction by a complexed poly-ribonucleotide. *Proc. Soc. Exp. Biol. Med.* **133**: 1334–1341.

TIMKOVSKY, A. L., AKSENOV, O. A., BRESLER, S. E., KOGAN, E. M., SMORODINTSEV, A. A. and TIKHOMIROVA-SIDOROVA, N. S. (1973) Molecular weight characteristics of the poly(G)·poly(C) complex and their relationship to the interferon-inducing and antiviral activities. *Vop. Virus* **18**: 350–355.

TOPAL, M. D. and FRESCO, J. R. (1976) Complementary base pairing and the origin of substitution mutations. *Nature* **263**: 285–289.

TORELLI, U., CADOSSI, R., TORELLI, G., FERRARI, ST. and FERRARI, SE. (1975) Inhibition of protein synthesis in reticulocyte lysates by double-stranded ribonucleic acid extracted from nuclei of leukemic cells. *FEBS Lett.* **56**: 96–100.

TORELLI, U., FERARI, ST., TORELLI, G., CADOSSI, R. and FERRARI, S. (1976) Double-stranded ribonuclease activity in human lymphocyte nuclei. *Experientia* **32**: 285–287.

TORRENCE, P. F. and DE CLERCQ, E. (1977) The polyadenylic-polyxanthylic-polyuridylic acid triple-helix. *Biochemistry* **16**: 1039–1043.

TORRENCE, P. F., DE CLERCQ, E., WATERS, J. A. and WITKOP, B. (1974) A potent interferon inducer derived from poly(7-deazainosinic acid) *Biochemistry* **13**: 4400–4408.

TORRENCE, P. F., DE CLERCQ, E., WATERS, J. A. and WITKOP, B. (1975a) Failure of duplexes based on polylaurusin [poly(L), 'polyformycin B'] to induce interferon. *Biochem. biophys. Res. Commun.* **62**: 658–664.

TORRENCE, P. F., DE CLERCQ, E. and WITKOP, B. (1975b) In search of the ideal polynucleotide inducer of interferon. *Proceedings of the Stanford Symposium on Antivirals with Clinical Potential.* Stanford, California, abstract # IId.

TORRENCE, P. F., DE CLERCQ, E. and WITKOP, B. (1976a) Triple-helical polynucleotides. Mixed triplexes of the poly(uridylic acid)·poly(adenylic acid)·poly(uridylic acid) class. *Biochemistry* **15**: 724–734.

TORRENCE, P. F., DE CLERCQ, E. and WITKOP, B. (1977) The interaction of polyadenylic acid with polyxanthylic acid. *Biochim. biophys. Acta* **475**: 1–6.

TORRENCE, P. F., WATERS, J. A., BUCKLER, C. E. and WITKOP, B. (1973) Effect of pyrimidine and ribose modifications on the antiviral activity of synthetic polynucleotides. *Biochem. biophys. Res. Comm.* **52**: 890–898.

TORRENCE, P. F. and WITKOP, B. (1975) Polynucleotide duplexes based on poly(7-deazaadenylic acid). *Biochim. biophys. Acta* **395**: 56–66.

TORRENCE, P. F. and WITKOP, B. (1976) Design of polynucleotides that act as inducers of interferon. (in Japanese) *Protein, Nucleic Acid, and Enzyme.* **21**: 536–543.

TOVELL, D. and CANTELL, K. (1971) Kinetics of interferon production in human leukocyte suspensions. *J. gen. Virol.* **13**: 485–489.

TS'O, P. O. P., ALDERFER, J., COCHRAN, D., GREENE, J., MORRY, D., CARTER, W., O'MALLEY, J., HOROSZEWICZ, J. and MARSHALL, L. (1975) Relation between primary structure and biological activities of poly(I)·poly(C). *Abstracts of the 75th Annual Meeting of the American Society for Microbiology*, p. 269, number S 334.

TS'O P. O. P., ALDERFER, J. L., LEVY, J., MARSHALL, L. W., O'MALLEY, J., HOROSZEWICZ, J. S. and CARTER, W. A. (1976) An integrated and comparative study of the antiviral effects and other biological properties of the polyinosinic acid-polycytidylic acid and its mismatched analogues. *Molec. Pharmac.* **12**: 299–312.

TURNER, W., CHAN, S. P. and CHIRIGOS, M. A. (1970) Stimulation of humoral and cellular antibody formation in mice by I_n:C_n. *Proc. Soc. exp. Biol. Med.* **133**: 334–338.

TYTELL, A. A. and FIELD, A. K. (1972) Interferons and host resistance: with particular emphasis on induction by complexed polynucleotides. *CRC Crit. Rev. Biochem.* **1**: 1–30.

TYTELL, A. A., LAMPSON, G. P., FIELD, A. K. and HILLEMAN, M. R. (1967) Inducers of interferon and host resistance, III. Double-stranded RNA from reovirus type 3 virions (Reo 3-RNA). *Proc. natn. Acad. Sci. USA* **58**: 1719–1722.

TYTELL, A. A., LAMPSON, G. P., FIELD, A. K., NEMES, M. M. and HILLEMAN, M. R. (1970) Influence of size of individual homopolynucleotides on the physical and biological properties of rI_n:rC_n (poly I:C). *Proc. Soc. exp. Biol. Med.* **135**: 917–921.

UCHIC, J. T. (1975) Potential interferon-inducers. Complexes of poly(3-isoadenylic acid). *Abstracts of the 170th National meeting of the American Chemical Society, abstract no. MEDI 43.*

USTACELEBI, S. and WILLIAMS, J. F. (1972) Temperature-sensitive mutants of adenovirus defective in interferon induction at non-permissive temperature. *Nature* **235**: 52–53.

USTACELEBI, S. and WILLIAMS, J. F. (1973) Induction of interferon in chick cells by polyoma virus. *J. gen. Virol.* **21**: 163–168.

VALLE, M. J., BOBGROVE, A. M., STROBER, S. and MERIGAN, T. C. (1975a) Immune specific production of interferon by human T cells in combined macrophage-lymphocyte cultures in response to herpes simplex antigen. *J. Immunol.* **114**: 435–441.

VALLE, M. J., JORDAN, G. W., HAAHR, S. and MERIGAN, T. C. (1975b) Characteristics of immune interferon produced by human lymphocyte cultures compared to other human interferon. *J. Immunol.* **115**: 230–233.

VAN DUCK, P. J., CLAESEN, M. and DE SOMER, P. (1970) Effect of polyacrylic acid on experimental malaria and trypanosomiasis in mice. *Ann. Trop. Med. Parasit.* **64**: 5–9.

VENGRIS, V. E., STOLLAR, B. D. and PYTHA, P. M. (1975) Interferon externalization by producing cell before induction of antiviral state. *Virology* **65**: 410–417.

VILCEK, J. (1962) Studies on an interferon from tick-borne encephalitis virus-infected cells, IV. Comparison of IF with interferon from influenza virus-infected cells. *Acta Virol.* **6**: 144–150.

VILCEK, J. (1970a) Metabolic determinants of the induction of interferon by a synthetic double-stranded polynucleotide in rabbit kidney cells. *Ann. N.Y. Acad. Sci.* **173**: 390–403.

VILCEK, J. (1970b) Cellular mechanisms of interferon production. *J. gen. Physiol.* **56**: 76*s*–89*s*.

VILCEK, J., BARMAK, S. L. and HAVELL, H. A. (1972) Control of interferon synthesis: effect of diethylaminoethyl-dextran on induction by polyinosinic-polycytidylic acid. *J. Virology* **10**: 614–621.

VILCEK, J. and HAVELL, E. A. (1973) Stabilization of interferon messenger RNA activity by treatment of cells with metabolic inhibitors and lowering of the incubation temperature. *Proc. natn. Acad. Sci. USA* 70: 3909–3913.

VILCEK, J., HAVELL, E. A., MOZES, L. W. and BERMAN, B. (1975) Interferon induction with viruses and polynucleotides. In: *Proceedings of the First Intersectional Congress of The International Association of Microbiological Societies*, pp. 65–75, Vol. 4, HASEGAWA, T. (ed.). Science Council of Japan.

VILCEK, J. and NG, M. H. (1971) Post-transcriptional control of interferon synthesis. *J. Virol.* **7**: 588–594.

VILCEK, J., NG, M. H., FRIEDMAN-KIEN, A. E. and KRAWCIW, T. (1968) Induction of interferon synthesis by synthetic double-stranded polynucleotides. *J. Virol.* **2**: 648–650.

VILCEK, J. and RADA, B. (1962) Studies on an interferon from tick-borne encephalitis virus-infected cells, III. Antiviral action of IF. *Acta Virol.* **6**: 9–16.

VILCEK, J., ROSSMAN, T. G. and VARACALLI, F. (1969) Differential effects of actinomycin D and puromycin on the release of interferon induced by double-stranded RNA. *Nature* **222**: 682–683.

VILCEK, J. and VARACALLI, F. (1971) Sequential suppression by actinomycin D of interferon production and cellular resistance induced by poly I:C. *J. gen. Virol.* **13**: 185–188.

VON HIPPEL, P. H. and MCGHEE, J. D. (1972) DNA-protein interactions. *A. Rev Biochem.* **41**: 231–300.

WACKER, A., LODEMAN, E., DIEDERICH, J., MOHRBUTTER, K. and LARISCHKE, U. (1972) Induction of interferon in L-cells by polyinosinic-polycytidylic acid in the presence of cationic compounds. I. Polycations. *Arch. Ges. Virusforsch.* **36**: 71–79.

WACKER, A., SINGH, A., SVEC, J. and LODEMAN, E. (1969) Influence of chain length of oligonucleotides on interferon induction. *Naturwissenschaften* **56**: 638.

WAGNER, A. R., BUGIANESI, R. L. and STERN, T. Y. (1971) Preparation of sepharosebound poly (rI:rC). *Biochem. biophys. Res. Commun.* **45**: 184–189.

WALLEN, W. C., DEAN, J. H. and LUCAS, D. O. (1973) Interferon and the cellular immune response: separation of interferon-producing cells from DNA-synthetic cells. *Cell. Immunol.* **6**: 110–122.

WANG, A. C. and KALLENBACH, N. R. (1971) Helical complexes of polyriboinosinic acid with copolymers of polyribocytidylic acid containing inosine, adenosine and uridine residues. *J. molec. Biol.* **62**: 591–611.

WATSON, J. D. (1976) *Molecular Biology of The Gene*, pp. 347–376, 3rd edn, Benjamin, California.

WEINSTEIN, J. J., WAITZ, J. A. and CAME, P. E. (1970) Induction of resistance to bacterial infections of mice with poly I·poly C. *Nature* **226**: 170.

WHEELOCK, E. F. (1965) Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. *Science* **149**: 310–311.

WICKNER, R. B. (1976) Killer of *Saccharomyces cerevisiae*: a double-stranded ribonucleic acid plasmid. *Bacteriol. Rev.* **40**: 757–773.

WOODS, W. A., SIEGEL, M. I. and CHIRIGOS, M. A. (1974) *In vitro* stimulation of spleen cell cultures by poly I:poly C and levamisole. *Cell. Immunol.* **14**: 327–331.

YAMADA, M., AZUMA, M., NISHIOKA, R. and TOGASHI, T. (1970) Relationship between immunity and interferon production in macrophages, I. Effect of immunity on interferon production. *Jap. J. Microbiol.* **14**: 311–318.

YARUS, M. (1969) Recognition of nucleotide sequences. *A. Rev. Biochem.* **38**: 841–880.

YOUNG, C. W. (1971) Interferon induction in cancer with some observations on the clinical effects of poly I:C. *Med. Clin. North Am.* **55**: 721–728.

YOUNGNER, J. S. (1970a) Interferon production by nonviral stimuli of microbial origin. *J. gen. Physiol.* **56** (supplement): 25s–38s.

YOUNGNER, J. S. (1970b) Influence of inhibitors of protein synthesis on interferon formation in mice. II. Comparison of effects of glutarimide antibiotics and tenuazonic acid. *Virology* **40**: 335–343.

YOUNGNER, J. S. (1972) Bacterial lipopolysaccharide: oral route for interferon production in mice. *Infect. Immunity* **6**: 646–647.

YOUNGNER, J. S. and FEINGOLD, D. S. (1967) Interferon production in mice by cell wall mutants of *Salmonella typhimurium*. *J. Virol.* **1**: 1164–1167.

YOUNGNER, J. S., FEINGOLD, D. S. and CHEN, J. K. (1973) Involvement of a chemical moiety of bacterial lipopolysaccharide in production of interferon in animals. *J. Infect. Dis.* **128** (supplement): S227–S231.

YOUNGNER, J. S. and HALLUM, J. V. (1968) Interferon production in mice by double-stranded polynucleotides: induction or release. *Virology* **35**: 177–179.

YOUNGNER, J. S. and HALLUM, J. V. (1969) Inhibition of induction of interferon synthesis in L cells pre-treated with interferon. *Virology* **37**: 473–475.

YOUNGNER, J. S., KELETI, G. and FEINGOLD, D. S. (1974) Antiviral activity of an ether-extracted nonviable preparation of *Brucella abortus*. *Infect. Immunity* **10**: 1202–1206.

YOUNGNER, J. S. and SALVIN, S. B. (1973) Production and properties of migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. *J. Immunol.* **111**: 1914–1922.

YOUNGNER, J. S. and STINEBRING, W. R. (1964) Interferon production in chickens injected with *Brucella abortus*. *Science* **144**: 1022–1023.

YOUNGNER, J. S. and STINEBRING, W. R. (1965) Interferon appearance stimulated by endotoxin, bacteria, or viruses in mice pretreated with *Escherichia coli* endotoxin or infected with *Mycobacterium tuberculosis*. *Nature* **208**: 456–458.

YOUNGNER, J. S. and STINEBRING, W. R. (1966) Comparison of interferon production in mice by bacterial endotoxin and statolon. *Virology* **29**: 310–316.

YOUNGNER, J. S., STINEBRING, W. R. and TAUBE, S. E. (1965) Influence of inhibitors of protein synthesis on interferon formation in mice. *Virology* **27**: 541–550.

ZIMMERMAN, S. B., COHEN, G. H. and DAVIES, D. R. (1975) X-ray fiber diffraction and model-building study of polyguanylic acid and poly-inosinic acid. *J. molec. Biol.* **92**: 181–192.

MOLECULAR AND CELLULAR BIOLOGY, Nov. 1992, p. 5238–5248
0270-7306/92/115238-11$02.00/0

Vol. 12, No. 11

Interactions between Double-Stranded RNA Regulators and the Protein Kinase DAI

LISA MANCHE, SIMON R. GREEN, CHRISTIAN SCHMEDT, AND MICHAEL B. MATHEWS*
Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received 6 May 1992/Returned for modification 2 July 1992/Accepted 27 August 1992

The interferon-induced protein kinase DAI, the double-stranded RNA (dsRNA)-activated inhibitor of translation, plays a key role in regulating protein synthesis in higher cells. Once activated, in a process that involves autophosphorylation, it phosphorylates the initiation factor eIF-2, leading to inhibition of polypeptide chain initiation. The activity of DAI is controlled by RNA regulators, including dsRNA activators and highly structured single-stranded RNAs which block activation by dsRNA. To elucidate the mechanism of activation, we studied the interaction of DAI with RNA duplexes of discrete sizes. Molecules shorter than 30 bp fail to bind stably and do not activate the enzyme, but at high concentrations they prevent activation by long dsRNA. Molecules longer than 30 bp bind and activate the enzyme, with an efficiency that increases with increasing chain length, reaching a maximum at about 85 bp. These dsRNAs fail to activate at high concentrations and also prevent activation by long dsRNA. Analysis of complexes between dsRNA and DAI suggests that at maximal packing the enzyme interacts with as little as a single helical turn of dsRNA (11 bp) but under conditions that allow activation the binding site protects about 80 bp of duplex. When the RNA-binding site is fully occupied with an RNA activator, the complex appears to undergo a conformational change.

Protein synthesis is modulated at several levels, most commonly at the stage of polypeptide chain initiation, and the phosphorylation of initiation factors plays a key role in controlling this process (reviewed in references 19 and 20). In mammalian cells, a regulatory mechanism involving an RNA-activated protein kinase and the eukaryotic initiation factor 2 (eIF-2) has been intensively studied. This initiation factor forms a ternary complex with GTP and Met-tRNA$_F$ and delivers the initiator tRNA to the ribosomal site of protein synthesis initiation. Discharged eIF-2 is subsequently released as a complex with GDP which must be replaced with GTP to permit the formation of another ternary complex in preparation for a further round of initiation. The factor is composed of three dissimilar subunits, α, β, and γ. Phosphorylation of a single residue, serine-51 of the α subunit, inhibits translation by trapping a second initiation factor, the guanosine nucleotide exchange factor (or eIF-2B), which is required to catalyze the substitution of GTP for GDP in the discharged eIF-2 complex. Phosphorylation of sufficient eIF-2 can sequester all of the guanosine nucleotide exchange factor, thereby preventing eIF-2 recycling and halting the initiation pathway.

In mammals, two protein kinases are capable of phosphorylating the α subunit of eIF-2 in this way (reviewed in references 20, 37, and 46). One of them, the heme-controlled repressor, is found chiefly in reticulocytes. It is activated by the absence of hemin, as well as by other stimuli, and serves to prevent the accumulation of globin in the absence of iron or heme. A second kinase, the double-stranded RNA-activated inhibitor (DAI; also referred to as P1 kinase, p68 kinase, P1/eIF-2α kinase, and PK$_{ds}$, etc.) is present in a wide range of tissues. DAI is an important element in the host antiviral response, and its synthesis is induced at the transcriptional level by interferon (reviewed in references 21, 54, 56, and 59). The enzyme is ribosome associated (11, 34) and normally exists in an inactive or latent state. Under some circumstances, DAI activation leads to the virtually complete abrogation of protein synthesis, while in other circumstances it may contribute to the selective translation of particular classes of mRNA (8, 24, 26, 36, 47, 60). It has also been implicated in cellular differentiation (23, 52), in the inhibition of cell proliferation (6, 51), in the heat shock response (10), and possibly in transcriptional induction (61, 64). Moreover, in yeast cells, the related protein kinase GCN2 mediates the growth response to amino acid starvation (9). As its name implies, DAI is activated by double-stranded RNA (dsRNA). Other polyanions such as heparin can also activate it, while small, highly structured RNA molecules such as adenovirus VA RNA suppress its activation (38). Thus, DAI is a pivotal cellular regulatory enzyme whose level and activity are modulated by factors of both viral and cellular origin.

The interactions between DAI and its RNA effectors are complicated and incompletely understood. The kinase is activated by dsRNA but not by DNA or DNA-RNA hybrids (22, 32, 35, 58). Single-stranded RNA, either synthetic or natural, is also inactive unless it can form extended hairpin-like structures (5, 22). There is no discernible sequence dependence for activation by dsRNA, and limited mismatching (44) and some modified bases (2, 45, 62) are tolerated, but the activity of dsRNA is reduced by ethidium bromide (1), suggesting that the topological form of the RNA duplex is important. Activation is accompanied by autophosphorylation of the kinase at multiple sites on serine and threonine residues (3, 11, 14, 30), and results in a change of substrate specificity such that the activated enzyme can phosphorylate the α subunit of eIF-2 and some other proteins (53, 58) but can no longer phosphorylate other molecules of DAI (29). Once activated, however, the phosphorylated enzyme is unaffected by the addition or removal of dsRNA (11, 58, 63).

Activation of DAI by dsRNA displays a paradoxical concentration dependence: the enzyme is activated by low concentrations of dsRNA (in the range of 10 to 100 ng/ml), but higher concentrations are decreasingly effective activators, giving rise to a bell-shaped activation curve (11, 22, 27,

* Corresponding author.

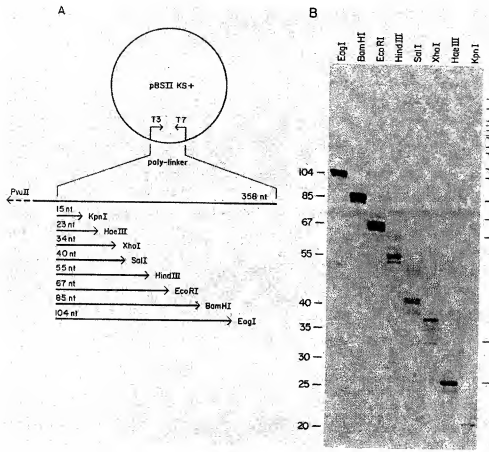


FIG. 1. Synthesis and characterization of RNA duplexes. (A) Schematic of short dsRNAs produced by transcription of pBSII KS+ polylinker sequences. The several transcripts of 15 to 104 nt synthesized by T3 RNA polymerase (rightward arrows) were annealed to the complementary 358-nt transcript (leftward arrows) synthesized by T7 RNA polymerase. After RNase digestion, the duplexes were purified by electrophoresis in nondenaturing gels. (B) Analysis of the purified dsRNAs in denaturing conditions. Samples of the radiolabeled dsRNAs were heated in formamide and resolved by electrophoresis in a 10% polyacrylamide–7 M urea gel. The fixed and dried gel was subjected to autoradiography. Size markers were single-stranded RNAs synthesized as described in part A (left) and pBR322-*Hpa*II DNA fragments (right).

33). High concentrations of dsRNA prevent the activation process but do not interfere with the activity of DAI once it has been activated by dsRNA at a lower concentration. The kinase also displays a stringent requirement for dsRNA chain length. Activation is reported to require a minimum of about 50 bp of duplex (18, 22, 32, 44), and there are indications that shorter duplexes may block activation at high concentration (44), as long dsRNA does. The ability of the enzyme to discriminate between dsRNA molecules on the basis of their chain length has implications for its regulation and the mechanism of DAI activation (37). Here we investigate the interactions of the enzyme with dsRNA molecules of specified sizes, studying binding and protection of dsRNA as well as activation and inhibition of the kinase. Our results define the dsRNA size dependence of the interaction and confirm that short duplexes which fail to bind stably and to activate the kinase can still interfere with activation mediated by longer duplexes. The data suggest that the minimal recognition element is a single helical turn but that there is an extended site for dsRNA binding which needs to be completely occupied for full enzyme activation.

MATERIALS AND METHODS

Synthesis of short dsRNAs. The plasmid pBSII KS+ (Stratagene, Inc., La Jolla, Calif.) was banded twice in CsCl, passed over a Bio-Gel A 15-m column, and then digested with one of eight enzymes (*Kpn*I, *Hae*III, *Xho*I, *Sal*I, *Hin*dIII, *Eco*RI, *Bam*HI, or *Eag*I), which cut in the polylinker, or with *Pvu*II, which cuts outside the region containing the polylinker and the T3 and T7 promoters (Fig. 1A). The DNA was incubated with RNase A to remove the last traces of RNA, treated with proteinase K, and extracted with phenol and chloroform. After ethanol precipitation, the DNA was added to transcription reactions containing T7 RNA polymerase (17) for the PvuII-digested template or T3 RNA polymerase (Stratagene, Inc.) for the other templates. Reaction conditions were as described previously (43), except that the concentration of GTP or CTP was reduced to 12 μM for labeling. The corresponding [α-^{32}P]ribonucleotide (from ICN Biomedicals Inc., Costa Mesa, Calif.) was present at a concentration of 500 μCi/ml. Single-stranded RNA was recovered after DNaseI digestion and phenol and chloroform extraction by ethanol precipitation. Each of the

T3 products (15 to 104 nucleotides [nt]) was mixed with an approximately equivalent amount of the complementary T7 product (354 nt), heated to 100°C, and annealed as previously described (29). Following digestion with both RNase T_1 and RNase A, dsRNA was isolated by treatment with proteinase K and deproteinization and then fractionated by electrophoresis in a nondenaturing 10% polyacrylamide–0.5× Tris-borate-EDTA (TBE) gel. The bands were detected autoradiographically, and each dsRNA was eluted into 10 mM Tris–1 mM EDTA–10 mM NaCl–0.5% sodium dodecyl sulfate (SDS), deproteinized, and ethanol precipitated. The dsRNA was dissolved in the same buffer without SDS, and its concentration was calculated from the specific activity.

Other RNAs. Longer dsRNAs (354 bp) were synthesized by transcription of the pGEM.GC plasmid (42). Reovirus dsRNA was provided by A. J. Shatkin (Rutgers University, New Brunswick, N.J.), and *Penicillium chrysogenum* and bacteriophage f2 sus3 dsRNAs were provided by H. D. Robertson (Cornell University Medical School, Ithaca, N.Y.). Labeled single-stranded RNA was purified from the T7 and T3 polymerase transcription reactions described above by electrophoresis through a 10% polyacrylamide–7 M urea–0.5× TBE gel.

Kinase assays. Reactions (10 μl) containing 2.5 μCi of [γ-^{32}P]ATP (ICN Biomedicals, Inc.) and 0.5 μl of DAI (about 5 ng) purified to the Mono S stage (29) were conducted essentially as described by Mellits et al. (42). The enzyme was added last to the other reaction components assembled on ice. Phosphorylation was visualized by SDS-polyacrylamide gel electrophoresis and autoradiography for 4 to 16 h by using an intensifier screen.

Nitrocellulose filter-binding assay. The nitrocellulose filter-binding assay was conducted by using a modification of the published procedure of Kostura and Mathews (29). Briefly, labeled dsRNA was incubated for 20 min on ice with the Mono S fraction of DAI under kinase reaction conditions, with bovine serum albumin (BSA) and calf liver tRNA both added to a concentration of 0.1 mg/ml but with labeled ATP omitted. After dilution with 10 volumes of wash buffer (50 mM KCl, 1.5 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) K^+ [pH 7.4], 0.1 mM EDTA), the reaction mixtures were immediately filtered in a slot-blot apparatus through a 0.45-μm-pore-size nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) that had been soaked for 1 h at room temperature in wash buffer containing 0.1 mg each of BSA and salmon sperm DNA per ml. Each well was washed with 200 μl of ice-cold wash buffer, and the filter was dried and exposed to autoradiography. Quantitation was done by scintillation counting of individual bands or direct scanning of the membrane with the AMBIS Imaging System.

Binding of dsRNA to Sepharose-bound DAI. A mixture of dsRNAs was partially degraded by incubation with RNase T_1 and RNaseIII (provided by H. D. Robertson) and then incubated with DAI immobilized on monoclonal antibody-Sepharose beads (13, 31) (from A. Hovanessian, Institut Pasteur, Paris, France) as described previously (40). The beads were sedimented and washed five times by resuspension and sedimentation. Of the input radioactivity, approximately 15% was recovered with the beads, 80% was recovered in the initial supernatant plus first wash fraction and 5% was recovered in subsequent washes. RNA was extracted from the beads and from the initial supernatant plus the first wash fraction and was analyzed by electrophoresis in a nondenaturing 10% polyacrylamide–0.5× TBE gel.

Protection of dsRNA by DAI. Radiolabeled dsRNA (354 bp) was bound to immobilized DAI as described above. After the third wash, the beads were washed twice with RNaseIII buffer (100 mM NH_4Cl, 10 mM magnesium acetate, 20 mM Tris-HCl [pH 7.6]) and then incubated with 50 U of RNaseIII per ml for 30 min at 37°C. An equal amount of fresh RNaseIII was added, and the incubation continued for a further 30 min. RNA was isolated from the beads and from the supernatant fractions and analyzed as described above.

Gel retardation assay. Binding reactions (10 μl) were similar to those for kinase assays, except that ATP was omitted and tRNA and BSA were present at 0.1 and 1 mg/ml, respectively. The concentration of labeled dsRNA was 55 ng/ml, and the concentration of DAI (Mono S fraction), immunoaffinity chromatography-purified DAI (14), or p20 (55) was varied. After incubation for 20 min on ice, a dye-glycerol solution was added and the samples were loaded directly onto a 5% polyacrylamide gel (acrylamide: bioacrylamide, 82:1). The gel was cast in 40 mM Tris-glycine buffer and had been prerun for 1 h at 150 V. Radioactivity was detected by autoradiography for approximately 16 h.

RESULTS

Characteristics of synthetic dsRNA. Duplexed RNAs of defined sizes were made by annealing a 358-nt transcript synthesized by T7 RNA polymerase with complementary transcripts of various lengths synthesized by T3 RNA polymerase (Fig. 1A). After digestion of the RNA tails and residual single-stranded RNA, the dsRNAs were purified by electrophoresis in nondenaturing polyacrylamide gels. When analyzed in denaturing conditions (Fig. 1B), the individual strands of the dsRNA molecules were slightly heterogeneous, with chain lengths a few nucleotides longer or shorter than the input single strands as a result of the trimming process. When examined in a nondenaturing gel, however, the dsRNAs migrated as discrete bands, with mobilities similar to those of dsDNA markers (see Fig. 5A, lanes 3 to 9). As expected, the duplexes were sensitive to digestion with RNaseIII, a dsRNA-specific enzyme, but resistant to digestion by single-stranded specific nucleases except after denaturation (data not shown).

Activation and inhibition of DAI. Activation of DAI is accompanied by its autophosphorylation, converting the enzyme from a latent state to a form which can phosphorylate eIF-2α. When the synthetic duplexes were examined for their ability to catalyze autophosphorylation, we found that 23- and 34-bp dsRNAs were only slightly active, 40-bp dsRNA was partly active, and full activity was approached with 55- to 85-bp dsRNAs, which were nearly as active as the very long dsRNA (average size of >2,000 bp) isolated from reovirus virions (Fig. 2A). These results, obtained with essentially flush-ended dsRNA, agree closely with previously reported data obtained by using RNA molecules in which one strand was considerably longer than the other (44): in the earlier study, duplexes shorter than 30 bp were unable to activate DAI, and full activation was obtained with duplexes longer than 65 to 80 bp. The activation of DAI can also be monitored by phosphorylation of eIF-2, the natural substrate of this kinase. In this assay, 15-bp dsRNA was essentially inactive, 34-bp dsRNA was partially active, and 55-bp (or longer) dsRNA was fully active (Fig. 2B). Thus, the slight autophosphorylation of DAI that is catalyzed by the 34-bp duplex is sufficient to permit DAI to phosphorylate its natural substrate weakly. These results are consistent with the findings that the very short (<20 bp) imperfect duplexes found in viral RNAs such as VA RNA (28, 38, 41,

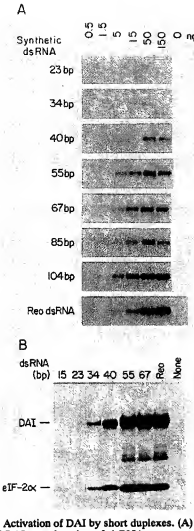


FIG. 2. Activation of DAI by short duplexes. (A) Autophosphorylation of DAI as a function of dsRNA concentration and chain length. Kinase assay reaction mixtures containing synthetic or reovirus dsRNA at the concentrations indicated were analyzed by electrophoresis in SDS-polyacrylamide gels and autoradiography. (B) Phosphorylation of eIF-2. Kinase assay reaction mixtures containing 33 ng of the synthetic dsRNAs indicated per ml or 40 ng of reovirus dsRNA per ml were supplemented with eIF-2 and were analyzed as described in part A. The positions of autophosphorylated DAI and the phosphorylated α subunit of eIF-2 are marked.

42, 48), EBER (4, 7) and TAR RNA (18) are insufficient to cause DAI activation.

From experiments with tailed duplexes, it was concluded that short dsRNAs which are incapable of activating DAI may nevertheless block the activation of DAI by longer dsRNAs (44). Figure 3A shows that at high concentration a flush-ended 23-bp dsRNA inhibited the activation of DAI by reovirus dsRNA. In this respect, short dsRNA resembles longer dsRNA molecules which block DAI activation at high concentrations, although the mechanisms might be different. Figure 3B demonstrates that long dsRNA (approximately 3,000 bp) isolated from *P. chrysogenum* activates DAI at concentrations of up to 1 μg/ml and prevents activation at 10 μg/ml, as expected from the bell-shaped activation curve.

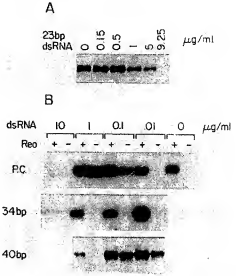


FIG. 3. Inhibition of DAI activation by high concentrations of dsRNA. (A) Inhibition of DAI autophosphorylation by 23-bp dsRNA. Kinase assay reaction mixtures contained 20 ng of reovirus dsRNA per ml and the indicated concentrations of the 23-bp dsRNA. (B) Effect of duplexes of various lengths on DAI autophosphorylation in the presence and absence of an activator dsRNA. Kinase assay reaction mixtures containing or lacking reovirus dsRNA (40 ng/ml) were supplemented with synthetic 34- or 40-bp dsRNAs or with *P. chrysogenum* dsRNA (P.C.) at the concentrations indicated. Autophosphorylation of DAI was assessed as described for Fig. 2.

Short duplexes, such as the 34-bp dsRNA (Fig. 3B), activate DAI only weakly and also become inhibitory at approximately 10 μg/ml. Similar results were obtained with bacteriophage f2 sus3 dsRNA (approximately 30 to 50 bp long [22]) and the 23-bp synthetic duplexes (data not shown), while the 40-bp dsRNA gave a response intermediate between that of the longer and shorter duplexes because of its significant ability to activate DAI (Fig. 3B). Single-stranded RNA is not inhibitory (data not shown). These results confirm that the enzyme is activated by relatively long dsRNA and can interact with short duplexes in a nonproductive way.

RNA size dependence of DAI binding. The existence of opposing effects of dsRNA on DAI activity makes it important to address directly the binding of this ligand to the protein. We examined the interactions between DAI and dsRNA, using three assays which explore different aspects of the process: nitrocellulose filter binding, binding to an affinity matrix, and gel retardation. Figure 4A and B illustrates the binding of single-stranded RNA and dsRNA as a function of chain length in the filtration assay. Duplexes with sizes of 15 and 23 bp did not bind detectably, whereas 34- and 40-bp duplexes bound weakly. Longer molecules bound with increasing efficiency, and 85- or 104-bp dsRNA bound as efficiently as 354-bp molecules. Single-stranded molecules bound only very weakly, except for the 354-nt molecule, which may be able to form a small amount of secondary structure. The binding of dsRNAs to DAI was also measured as a function of ligand concentration (Fig. 4C and D). The efficiency with which short duplexes bound to DAI did not improve at subsaturating RNA concentrations, suggesting that the affinity of the enzyme is low for molecules with sizes

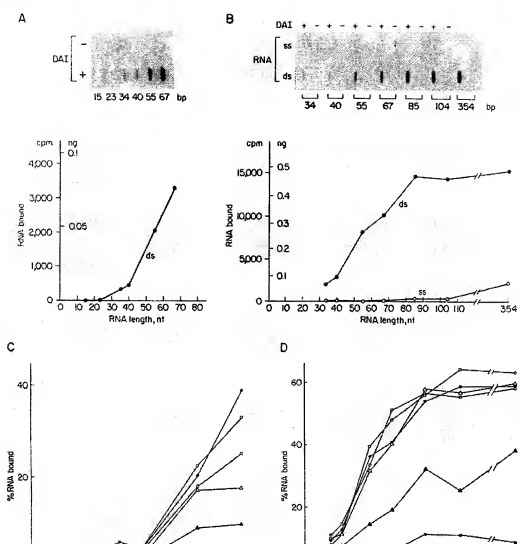


FIG. 4. Size dependence of dsRNA binding to DAI. (A and B) Nitrocellulose filter-binding assay for the binding of single-stranded RNA and dsRNAs. Autoradiograms of the membrane are shown (top panel). Quantitation of RNA binding (bottom panel) was obtained by scintillation counting or scanning of the individual filter bands and subtraction of the background value (lanes labeled −DAI) from the signal radioactivity (lanes labeled +DAI). ●, dsRNA (ds); ○, single-stranded RNA (ss). (C and D) Concentration and size dependence of dsRNA-binding efficiency. The percentage of the input dsRNA that was retained on the filter in the presence of DAI was quantified at various dsRNA concentrations of 1,000 (■), 330 (▲), 100 (△), 33 (□), 10 (○), and 3.3 (●) ng/ml, respectively.

of 40 bp or less and that the affinity increases uniformly as the chain length is increased, reaching a maximum at 85 bp. These data agree closely with the dependence of activation and inhibition on dsRNA chain length (Figs. 2 and 3) and are consistent with a model which equates activation with stable dsRNA binding. Duplexes shorter than 30 to 40 bp bind weakly and cannot activate although they inhibit activation; longer duplexes (40 to 85 bp) bind with increasing stability and their ability to activate the enzyme increases concomitantly; beyond this length, the efficiency of binding and activation remains unchanged.

Binding and protection of dsRNA. One interpretation of these observations is that the dsRNA binding site in DAI accommodates up to −85 bp of duplex but can bind shorter duplexes less stably, down to −30 bp. To test this interpretation we employed DAI immobilized on antibody-Sepharose beads. First, to define the minimum size of dsRNA that can bind to the enzyme, a mixture of dsRNA molecules was partially digested with RNaseIII to generate a collection of duplex molecules with a broad size distribution. This collection was allowed to bind to the immobilized DAI, and the beads were washed to remove nonspecifically adsorbed dsRNA. Figure 5A displays the DAI-bound dsRNA (lane 1) and the unbound RNA that remained in the supernatant (lane

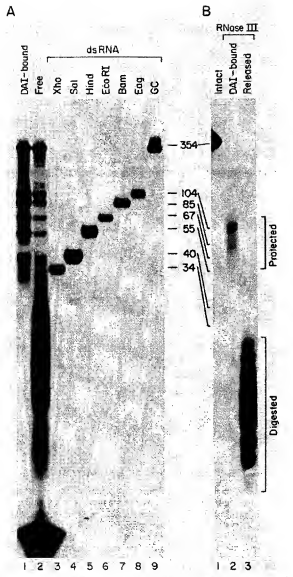


FIG. 5. Binding and protection of dsRNA fragments by DAI. (A) Binding of randomly sized dsRNA fragments. A mixture of fragments (approximately 10 to 350 bp) was incubated with DAI attached to Sepharose beads. Unbound RNA was separated from the beads by centrifugation and washing. Equal fractions of the DAI-bound (lane 1) and unbound (lane 2) dsRNA were resolved in a nondenaturing polyacrylamide gel and detected by autoradiography. Markers included discrete dsRNA fragments with sizes of 34 to 104 bp (lanes 3 to 8), denoted by the restriction site designation used in their synthesis (Fig. 1A), and 354 bp (lane 9), denoted GC for the plasmid used in its synthesis (pGEM.GC). (B) Protection of DAI-bound dsRNA from digestion by RNaseIII. Discrete 354-bp dsRNA (lane 1) was bound to DAI attached to Sepharose beads, and unbound RNA was removed. The beads were exhaustively incubated with RNaseIII, and the released RNA fragments were collected. Equal fractions of the released RNA (lane 3) and the RNA that remained associated with the beads (lane 2) were resolved as described for panel A.

2). Comparison with dsRNA markers (lanes 3 to 9) and with an RNA sequence ladder (data not shown), indicated that the cutoff for binding was at approximately 28 bp, in good agreement with results obtained in the nitrocellulose filter-binding assay. Moreover, visual inspection of the autoradiogram suggested that dsRNA with a size of 28 to 40 bp bound less efficiently than longer duplexes.

Next, we conducted a protection experiment to determine the length of dsRNA that is shielded by DAI from nuclease attack. Intact 354-bp dsRNA was bound to DAI immobilized on antibody-Sepharose beads, and the excess unbound dsRNA was removed. The bound dsRNA was digested by incubating the beads with RNaseIII to trim off regions of duplex that were not protected by DAI. Figure 5B, lane 3, shows that the released dsRNA had been reduced to fragments of approximately 10 to 20 bp as expected (57), whereas the bulk of the DAI-associated material (lane 2) ranged in size from approximately 60 to 120 bp, with a substantial concentration in the longer-size class (approximately 100 to 120 bp). Assuming that RNaseIII leaves 15 bp of dsRNA protruding on each side, we deduce that DAI associates with 30 to 90 bp of dsRNA. Taking 110 bp as the modal length of the protected fragments, it appears that about 80 bp of duplex interact directly with the enzyme, roughly the length of dsRNA that gives maximal binding in the nitrocellulose filter assay. The length of the protected fragment was not altered at relatively high concentrations of dsRNA (up to 1 μg/ml; data not shown), conditions which would be expected to disfavor oligomerization of DAI on the dsRNA. These findings support the view that the dsRNA site extends for ~80 bp and that shorter molecules bind with lesser affinity, provided that they are at least 28 bp long.

DAI-dsRNA complexes. To characterize the interactions more directly, we examined complexes formed between DAI and dsRNAs in a gel retardation assay (Fig. 6A). No complexes were observed with 15- or 23-bp duplexes (data not shown), but longer dsRNAs formed complexes with increasing efficiency. Four series of complexes were distinguishable (bands I to IV). Their relative abundance was principally a function of RNA chain length, with a lesser dependence on DAI concentration. On the basis of their behavior, the complexes seem to fall into two families. One family, containing the more slowly moving bands I and II, forms preferentially with duplexes of less than optimal length (34 to 67 bp) in binding and activation assays. The second family, containing the faster moving bands III and IV, forms preferentially with longer duplexes (≥85 bp), which are fully active in binding and activating DAI.

Band I was the most prominent complex with 55- and 67-bp duplexes but was barely detectable with longer or shorter duplexes. It was formed at low DAI concentrations and seemed to be converted to band II at elevated DAI concentrations. Indeed, regardless of chain length, complex II was seen only at high DAI concentrations. With 34- and 40-bp duplexes, the only detectable complexes appeared to migrate in band II. Formation of complex II increased as the chain length was extended, reaching maximal levels with the 67-bp duplex and declining as the chain length was extended further, to 85 and 104 bp.

The most abundant complexes, formed with 85- and 104-bp duplexes, migrated in band III. This band was also visible with 67-bp and perhaps 55-bp dsRNAs. As chain length increased, complex III was formed at progressively lower concentrations of DAI. It also seemed to decrease slightly at high DAI concentrations. Band IV displayed a pattern similar to that of band III but was always less

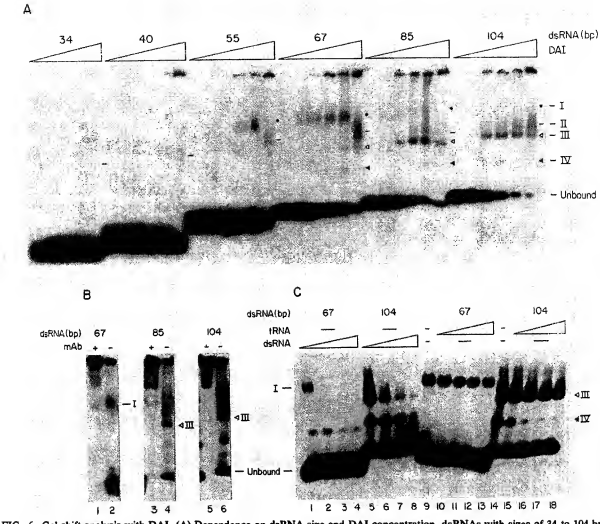


FIG. 6. Gel-shift analysis with DAI. (A) Dependence on dsRNA size and DAI concentration. dsRNAs with sizes of 34 to 104 bp were incubated with various amounts of DAI (0, 0.05, 0.1, 0.25, 0.5, and 1.0 μl from left to right, symbolized by the wedges) purified to the Mono S stage. The resultant complexes were separated by electrophoresis in nondenaturing conditions and detected by autoradiography. The positions of complexes I (●), II (—), III (◁), and IV (◀) and of the free dsRNA fragments are marked in each panel. (B) Shifts with essentially homogeneous DAI and antibody supershift. dsRNA with a size of 67 bp (lanes 1 and 2), 85 bp (lanes 3 and 4), or 104 bp (lanes 5 and 6) was incubated with 1 μl of DAI purified by immunoaffinity chromatography in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of 1 μl of monoclonal antibody to DAI. (C) Competition assays. Standard reaction mixtures (lanes 9 and 14) contained DAI (Mono S fraction) and 100 μg of tRNA per ml; *P. chrysogenum* dsRNA (0.25, 0.5, 0.75, and 1 μg/ml; lanes 1 to 4 and 5 to 8) or additional calf liver tRNA (100, 200, 300 and 400 μg/ml; lanes 10 to 13 and 15 to 18) was added as indicated. ^{32}P-labeled dsRNA (67 bp [lanes 1 to 4 and 9 to 13] or 104 bp [lanes 5 to 8 and 14 to 18]) was present at 55 ng/ml. The wedges symbolize increasing concentrations from left to right; — indicates absence of the RNA.

abundant. Both of these bands correlate well with full enzyme activity.

Very similar patterns of bands were formed with an essentially homogeneous preparation of DAI purified by immunoaffinity chromatography (Fig. 6B), and the gel-shift activity cosedimented with kinase activity through a glycerol gradient (29) (data not shown). Furthermore, as seen in Fig. 6B, all of the complexes were "supershifted" to forms with slower mobility by addition of monoclonal antibody directed against DAI. The antibody did not produce a gel shift on its own (i.e., in the absence of DAI), but it appeared to stabilize DAI-dsRNA complexes so that less probe remained in free form. These experiments verified that bands I to IV all contain DAI. Competition experiments demonstrated that the most prominent complexes, band I with 67-bp dsRNA and band III with 104-bp dsRNA, were resistant to the presence of excess tRNA but were sensitive to unlabeled *P. chrysogenum* dsRNA competitor (Fig. 6C). The complexes formed with the 104-bp dsRNA were more resistant to competition than those formed with 67-bp dsRNA, as expected from the higher affinity of DAI for longer duplexes (Fig. 4). For some of the minor bands (e.g., band IV), competition was more effective with tRNA at very high concentrations than with dsRNA at moderate concentrations

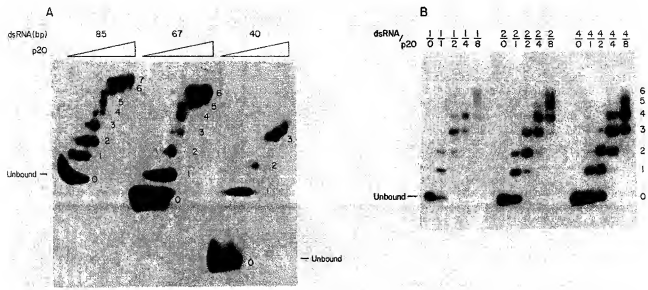


FIG. 7. Gel shift analysis with the p20 polypeptide. (A) Dependence on dsRNA size. Reaction mixtures contained dsRNA of 40, 67, or 85 bp as indicated and 0, 0.032, 0.063, 0.125, 0.25, 0.5, 2.5, 5, or 10 μg/ml of p20 (increasing from left to right as symbolized by the wedges). The number of p20 units in each band is indicated on the right. (B) Dependence on dsRNA and p20 concentration. Assay mixtures contained 85-bp dsRNA at a concentration of 55, 110, or 220 μg/ml and p20 at a concentration of 0, 63, 125, 250, or 500 ng/ml. Concentrations increase from left to right. The relative concentrations of these two components are indicated above the autoradiogram, and the number of p20 units in each band is marked on the side.

for reasons that are presently unclear. Neither single-stranded DNA nor dsDNA was an effective competitor (data not shown). These results demonstrate that the most prominent complexes are dsRNA and DAI specific and that there is a rather abrupt change in electrophoretic mobility when the dsRNA reaches the size for optimal binding and activation (approximately 80 bp).

Minimal binding site. The ability of short duplexes (<30 bp) to inhibit DAI activation implies that they interact with the enzyme, even though their binding is not sufficiently stable to be detected by the assays used to this point. To determine the minimal length of duplex that can interact with DAI, we employed the close-packing method for estimating the number of protein molecules that can bind to duplexes with known sizes. For this purpose, we used a truncated version of DAI, p20, comprising the N-terminal 184 amino acids which we and others have determined to contain the RNA-binding domain of the protein (16, 25, 39, 50). The numbers of p20 molecules binding to a given dsRNA were estimated from gel-shift assays conducted at increasing concentrations of the protein. As seen in Fig. 7A, a series of complexes was formed, reaching a maximum at the highest protein levels. The number of complexes increased with increasing dsRNA chain length as follows: 40 bp, three bands; 67 bp, six bands; 85 bp, seven bands. Assuming that each shifted band corresponds to the binding of a p20 molecule, these data imply that the minimum binding site is about 11 bp, equivalent to a single turn of A-form RNA helix.

The appearance of a ladder of bands with p20 suggested that there may be differences between the binding of this fragment and intact DAI to dsRNA. Further experiments showed that the formation of the p20 complexes is specific in that tRNA does not compete (data not shown). To rule out the possibility that the p20 banding patterns are due to a concentration-dependent protein oligomerization that is independent of dsRNA, we conducted band-shift assays at increasing dsRNA concentrations. Figure 7B shows that more p20 protein is required to achieve a given gel shift at higher concentrations of dsRNA (compare, for example, the amount of p20 needed to complex all of the dsRNA in the reactions). This indicates that oligomerization depends on the presence of dsRNA and is due to the formation of a series of protein-RNA complexes rather than to preformed protein-protein aggregates. The banding pattern was also influenced by the absolute concentration of p20 and dsRNA, however, as can be seen by comparing lanes with equal ratios of dsRNA to p20 (such as 1/1, 2/2, and 4/4). The shift to larger complexes at higher concentrations could merely reflect the concentration dependence of the reaction according to the law of mass action, or it could imply that p20 complexes are stabilized by protein-protein interactions when p20 monomers are bound adjacently on dsRNA. In the latter case, stabilizing protein-protein interactions would provide an explanation for the apparent paradox that DAI binds efficiently only to duplexes of longer than 30 bp but can bind to as little as a single helical turn of dsRNA.

DISCUSSION

Although the existence of DAI has been known for many years and its activation by a variety of polynucleotides has been studied intensively, an understanding of the enzyme's regulation has remained elusive. The kinase is activated by autophosphorylation in the presence of dsRNA. This response exhibits a number of unusual features: first, activation is prevented by high concentrations of dsRNAs which activate the kinase at low concentrations; second, short RNA duplexes fail to activate DAI at any concentration but prevent activation at elevated concentrations; third, highly structured single-stranded RNAs of viral origin also fail to activate DAI but can block activation by authentic, long

dsRNA. To illuminate the interactions between dsRNA and DAI, we generated a series of short RNA duplexes and studied directly their binding to the enzyme as well as their effects on its activity. The results correlate activation with the formation of stable complexes with a characteristic electrophoretic mobility and suggest a model that is compatible with the emerging understanding of DAI structure.

Our results are most consistent with the view that DAI possesses a single effective site for dsRNA, capable of accommodating approximately 80 bp of duplex. Two observations support this conclusion most strongly. First, as the length of the dsRNA ligand is increased, maximal binding is attained at this size and longer molecules bind no more efficiently, and second, the kinase protects this length of duplex from digestion by nuclease. Shorter duplexes, down to a lower limit of approximately 30 bp, bind with steadily decreasing efficiency while duplexes with lengths of less than 30 bp are unable to form a stable complex with DAI under normal conditions. Nonetheless, since such very short duplexes block the activation of DAI, we assume that at high concentrations they form transient interactions which prevent DAI activation. Likewise, other polynucleotides, such as RNA-DNA hybrids and partially methylated dsRNA duplexes that fail to activate the kinase, as well as long dsRNAs that can activate DAI, share this property of inhibiting kinase activation at high concentrations. The nature of these inhibitory interactions is unclear, and it remains to be seen whether viral effectors such as VA RNA, EBER, and TAR RNA function in the same way as short duplexes or whether they interact in a distinct fashion to block DAI activation. Preliminary data indicate that the sites for VA RNA and dsRNA are overlapping but perhaps not congruent (16).

How do these functional observations relate to the structure of the enzyme? DAI possesses two RNA-binding elements in its N-terminal domain (12, 16, 25, 39, 50). Each element contains an RNA-binding motif which is rich in basic amino acids and is predicted to form an α-helical structure (16, 39). Both elements are required for efficient binding of RNA, and they appear to cooperate to form a single bivalent site which optimally extends over approximately 80 bp of duplex. Since the RNA binding domain of DAI, expressed as the p20 protein, is able to interact with as little as 11 bp of dsRNA, we speculate that each element interacts with a single helical turn and that optimal binding occurs when these two turns are separated by about five intervening helical turns. In this complex, the entire span of approximately 80 bp is protected by DAI against attack by the dsRNA-specific nuclease RNase III. With this model, interactions with shorter dsRNA molecules entail increasing strain on the enzyme, accompanied by decreasing affinity, such that it becomes impossible for both elements to bind when there is less than one intervening helical turn (at approximately 33 bp). Evidently, monovalent complexes can also be formed at high ratios of enzyme to RNA as in the p20 gel-shift experiments: these complexes presumably involve only the stronger RNA binding region (region 1 [16]) and allow the protein to pack onto the RNA to a density of one molecule per helical turn.

According to this model, activation of the enzyme requires bivalent dsRNA binding which becomes detectable at approximately 30 bp and is most stable when the duplex is at least 80 bp long. Correlating with the formation of the most stable complexes is a shift in their mobility in the gel retardation assay. The predominant complex formed with dsRNA with a length of ≥85 bp is band III, which moves faster than the predominant complex formed with shorter dsRNA (band I). We considered the possibility that longer duplexes might be able to bind more DAI molecules than shorter duplexes, but because DAI is a basic protein (pI 8.6), it is unlikely that the acceleration in gel mobility that occurs between 67 and 85 bp with the shift from complex I to complex III is due to the binding of a second DAI molecule to a DAI-dsRNA complex. Therefore, we argue that the faster migration is probably due to a conformational change in the dsRNA or the DAI-dsRNA complex which leads to compaction and increased electrophoretic mobility. Compaction could result from relief of the distortion in DAI that occurs when the two binding elements can interact with optimally spaced sites on dsRNA. Alternatively, it could be accomplished if the RNA were bent or wrapped around the enzyme once it had filled the entire site. If this explanation is correct, it seems that the duplex must be continuous since an elevated concentration of 40-bp molecules does not have the same effect on binding or activation as an 80-bp duplex. The minor complexes, II and IV, seem to be related to complexes I and III, respectively, but display increased sensitivity to competition with tRNA. They are unlikely to represent the addition of a second molecule of DAI to a DAI-dsRNA complex because of the large retardation effect that this would be expected to have on electrophoretic mobility and there are few clues as to their structure or significance at present.

The proposal that DAI contains a single bipartite RNA-binding site provides an alternative to the two previous models for DAI activation, neither of which is readily compatible with the results presented here. The gel-shift data could be interpreted in terms of the model in which DAI possesses two distinct sites for dsRNA binding (15, 37), a high-affinity site for activation and a low-affinity inhibitory site, if it were supposed that duplexes with sizes of ≤67 bp bind at the inhibitory site whereas longer duplexes bind at the activating site. The resultant complexes could have significantly different mobilities. However, the apparent affinities for these duplexes are not greatly different in the binding assays shown here, so the postulate that the activation site is of much higher affinity than the inhibitory site is not satisfied. Moreover, 40- to 67-bp duplexes have significant ability to activate the enzyme. The data could also be interpreted in terms of the model that DAI is activated when two molecules bind to a single molecule of dsRNA (37, 49). On this basis, 80 bp would be the length of duplex required to span the RNA-binding sites of two DAI monomers. Each monomer would interact with 30 to 40 bp of dsRNA, and the complex would be stabilized by cooperative interactions between the protein molecules. The monotonous increase in binding efficiency with a chain length between 30 and 85 bp argues against this model, as does our failure to obtain protection of shorter RNA fragments at high ratios of DAI to dsRNA. Also, with this model, it is difficult to explain the multiplicity of complexes observed in band-shift experiments: in particular, longer duplexes or higher DAI concentrations would be expected to give rise to slower complexes, contrary to observations.

In summary, the data presented here suggest that DAI interacts with as little as 11 bp (one helical turn) of dsRNA, but activation is associated with the formation of a stable DAI-dsRNA complex. The formation of such a complex requires at least 30 bp of duplex (about three turns) and probably takes place when both of the enzyme's RNA-binding motifs are engaged with the ligand. Complex formation is optimal with dsRNA containing at least 80 bp (seven

to eight turns) and is apparently accompanied by a conformational change in the complex. We speculate that the bivalent interaction with dsRNA or the conformational change itself is critical for enzyme autophosphorylation and activation. With this model, short dsRNAs would be expected to block activation because they can interact with only one RNA-binding motif. Specific inhibitors of DAI activation, such as VA RNA, may also bind to one motif, or alternatively they may bind to both motifs but in such a way as to interfere with the conformation of the enzyme. Similarly, long dsRNAs that activate the enzyme at low concentrations might block activation at high concentrations because the two binding motifs form complexes with separate RNA duplexes, thereby precluding the requisite conformational change. While this is a satisfying explanation, there are alternatives which also fit the available facts. For example, since autophosphorylation appears to be intermolecular (29, 37), it is also possible that DAI serves as a phosphate acceptor only when it is not bound to dsRNA, a situation which would obtain at low or moderate concentrations of dsRNA. Consistent with this view, a truncated form of DAI that lacks the RNA binding site can still be phosphorylated by intact DAI (unpublished data). It is clear that further investigation will be required to establish the nature of the coupling between dsRNA binding and kinase activation: such studies are in progress.

ACKNOWLEDGMENTS

We thank P. Clarke, Y. Ma, K. Mellits, and T. Pe'ery for helpful discussions, A. Shatkin for reovirus dsRNA, A. Hovanessian for monoclonal antibody and antibody-Sepharose, and H. Robertson for *P. chrysogenum* dsRNA, f2 sus3 dsRNA, and RNase III.

Christian Schmedt was a member of the University of Konstanz/SUNY at Stony Brook exchange program. This work was supported by NIH grant CA13106.

REFERENCES

1. **Bagloni, C., and P. A. Maroney.** 1981. Inhibition of double-stranded ribonucleic acid activated protein kinase and 2',5'-oligo(adenylic acid) polymerase by ethidium bromide. Biochemistry **20**:758–762.
2. **Bagloni, C., M. A. Minks, and E. DeClercq.** 1981. Structural requirements of polynucleotides for the activation of (2'-5')A$_n$ polymerase and protein kinase. Nucleic Acids Res. **9**:4939–4950.
3. **Berry, M. J., G. S. Knutson, S. R. Laskey, S. M. Munemitsu, and C. E. Samuel.** 1985. Purification and substrate specificities of the double-stranded RNA-dependent protein kinase from untreated and interferon-treated mouse fibroblasts. J. Biol. Chem. **260**:11240–11247.
4. **Bhat, R. A., and B. Thimmappaya.** 1985. Construction and analysis of additional adenovirus substitution mutants confirm the complementation of VAI RNA function by two small RNAs encoded by Epstein-Barr virus. J. Virol. **56**:750–756.
5. **Bischoff, J. R., and C. E. Samuel.** 1989. Mechanism of interferon action. Activation of the human P1/eIF-2α protein kinase by individual reovirus s-class mRNAs: s1 mRNA is a potent activator relative to s4 mRNA. Virology **172**:106–115.
6. **Chong, K. L., L. Feng, K. Schappert, E. Meurs, T. F. Donahue, J. D. Friesen, A. G. Hovenessian, and B. R. G. Williams.** 1992. Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator *GCN2*. EMBO J. **11**:1553–1562.
7. **Clarke, P. A., N. A. Sharp, and M. J. Clemens.** 1990. Translational control by the Epstein-Barr virus small RNA EBER-1. Reversal of the double-stranded RNA-induced inhibition of protein synthesis in reticulocyte lysates. Eur. J. Biochem. **193**:635–641.
8. **deBenedetti, A., and C. Baglioni.** 1984. Inhibition of mRNA binding to ribosomes by localized activation of dsRNA-dependent protein kinase. Nature (London) **311**:79–81.
9. **Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. F. Donahue, and A. G. Hinnebusch.** 1992. Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. Cell **68**:585–596.
10. **Dubois, M.-F., J. Galabru, P. Lebon, B. Safer, and A. G. Hovanessian.** 1989. Reduced activity of the interferon-induced double-stranded RNA-dependent protein kinase during a heat shock stress. J. Biol. Chem. **264**:12165–12171.
11. **Farrell, P. J., K. Balkow, T. Hunt, and R. J. Jackson.** 1977. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. Cell **11**:187–200.
12. **Feng, G.-S., K. Chong, A. Kumar, and B. R. G. Williams.** 1992. Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. Proc. Natl. Acad. Sci. USA **89**:5447–5451.
13. **Galabru, J., and A. Hovanessian.** 1985. Two interferon-induced proteins are involved in the protein kinase complex dependent on double-stranded RNA. Cell **43**:685–694.
14. **Galabru, J., and A. Hovanessian.** 1987. Autophosphorylation of the protein kinase dependent on double-stranded RNA. J. Biol. Chem. **262**:15538–15544.
15. **Galabru, J., M. G. Katze, N. Robert, and A. G. Hovanessian.** 1989. The binding of double-stranded RNA and adenovirus VAI RNA to the interferon-induced protein kinase. Eur. J. Biochem. **178**:581–589.
16. **Green, S. R., and M. B. Mathews.** Submitted for publication.
17. **Grodberg, J., and J. J. Dunn.** 1988. *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. J. Bacteriol. **170**:1245–1253.
18. **Gunnery, S., A. P. Rice, H. D. Robertson, and M. B. Mathews.** 1990. Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **87**:8687–8691.
19. **Hershey, J. W. B.** 1989. Protein phosphorylation controls translation rates. J. Biol. Chem. **264**:20823–20826.
20. **Hershey, J. W. B.** 1991. Translational control in mammalian cells. Annu. Rev. Biochem. **60**:717–755.
21. **Hovanessian, A. G.** 1989. The double stranded RNA-activated protein kinase induced by interferon: dsRNA-PK. J. Interferon Res. **9**:641–647.
22. **Hunter, T., T. Hunt, R. J. Jackson, and H. D. Robertson.** 1975. The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. J. Biol. Chem. **250**:409–417.
23. **Judware, R., and R. Petryshyn.** 1991. Partial characterization of a cellular factor that regulates the double-stranded RNA-dependent eIF-2α kinase in 3T3-F442A fibroblasts. Mol. Cell. Biol. **11**:3259–3267.
24. **Katze, M. G., B. M. Detjen, B. Safer, and R. M. Krug.** 1986. Translational control by influenza virus: suppression of the kinase that phosphorylates the alpha subunit of initiation factor eIF-2 and selective translation of influenza viral messenger RNAs. Mol. Cell. Biol. **6**:1741–1750.
25. **Katze, M. G., M. Wambach, M.-L. Wong, M. Garfinkel, E. Meurs, K. Chong, B. R. G. Williams, A. G. Hovanessian, and G. N. Barber.** 1991. Functional expression and RNA binding analysis of the interferon-induced, double-stranded RNA-activated, 68,000-M_r protein kinase in a cell-free system. Mol. Cell. Biol. **11**:5497–5505.
26. **Kaufman, R. J., and P. Murtha.** 1987. Translational control mediated by eukaryotic initiation factor-2 is restricted to specific mRNAs in transfected cells. Mol. Cell. Biol. **7**:1568–1571.
27. **Kimchi, A., A. Zilberstein, A. Schmidt, L. Shulman, and M. Revel.** 1979. The interferon-induced protein kinase PK-i from mouse L cells. J. Biol. Chem. **254**:9846–9853.
28. **Kitajewski, J., R. J. Schneider, B. Safer, S. M. Munemitsu, C. E. Samuel, B. Thimmappaya, and T. Shenk.** 1986. Adenovirus VAI RNA antagonizes the antiviral action of the interferon by preventing activation of the interferon-induced eIF-2α kinase. Cell **45**:195–200.

29. **Kostura, M., and M. B. Mathews.** 1989. Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. Mol. Cell. Biol. 9:1576–1586.
30. **Lasky, S. R., B. L. Jacobs, and C. E. Samuel.** 1982. Characterization of the sites of phosphorylation in the interferon-induced phosphoprotein P1 from mouse fibroblasts: evidence for two forms of P1. J. Biol. Chem. 257:11067–11093.
31. **Laurent, A. G., B. Krust, J. Galabru, J. Svab, and A. G. Hovanessian.** 1985. Monoclonal antibodies to an interferon-induced M_r 68,000 protein and their use for the detection of double-stranded RNA-dependent protein kinase in human cells. Proc. Natl. Acad. Sci. USA 82:4341–4345.
32. **Lebleu, B., G. C. Sen, S. Shaila, B. Cabrer, and P. Lengyel.** 1976. Interferon, double-stranded RNA, and protein phosphorylation. Proc. Natl. Acad. Sci. USA 73:3107–3111.
33. **Lenz, J. R., and C. Baglioni.** 1978. Inhibition of protein synthesis by double-stranded RNA and phosphorylation of initiation factor, eIF-2. J. Biol. Chem. 253:4219–4223.
34. **Levin, D., and I. M. London.** 1978. Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2. Proc. Natl. Acad. Sci. USA 75:1121–1125.
35. **Levin, D. H., R. Petryshyn, and I. M. London.** 1980. Characterization of double-stranded-RNA-activated kinase that phosphorylates α subunit of eukaryotic initiation factor 2 (eIF-2α) in reticulocyte lysates. Proc. Natl. Acad. Sci. USA 77:832–836.
36. **Mathews, M. B.** 1991. Control of translation in adenovirus infected cells. Enzyme 44:250–264.
37. **Mathews, M. B., S. Gunnery, L. Manche, K. H. Mellits, and T. Pe'ery.** 1990. Control of protein synthesis by RNA regulators. NATO ASI (Adv Sci Inst) Ser H Cell Biol. 49:377–388.
38. **Mathews, M. B., and T. Shenk.** 1991. Adenovirus virus-associated RNA and translational control. J. Virol. 65:5657–5662.
39. **McCormack, S. J., D. C. Thomis, and C. E. Samuel.** 1992. Mechanism of interferon action: identification of a RNA binding domain within the N-terminal region of the human RNA-dependent P1/eIF-2α protein kinase. Virology 188:47–56.
40. **Mellits, K. H., M. Kostura, and M. B. Mathews.** 1990. Interaction of adenovirus VA RNA_I with the protein kinase DAI: non-equivalence of binding and function. Cell 61:843–852.
41. **Mellits, K. H., and M. B. Mathews.** 1988. Effects of mutations in stem and loop regions on the structure and function of adenovirus VA RNA_I. EMBO J. 7:2849–2859.
42. **Mellits, K. H., T. Pe'ery, L. Manche, H. D. Robertson, and M. B. Mathews.** 1990. Removal of double-stranded contaminants from RNA transcripts: synthesis of adenovirus VA RNA_I from a T7 vector. Nucleic Acids Res. 18:5401–5406.
43. **Meurs, E., K. Chong, J. Galabru, N. S. B. Thomas, I. M. Kerr, B. R. G. Williams, and A. G. Hovanessian.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell 62:379–390.
44. **Minks, M. A., D. K. West, S. Benvin, and C. Baglioni.** 1979. Structural requirements of double-stranded RNA for the activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. J. Biol. Chem. 254:10180–10183.
45. **Minks, M. A., D. K. West, S. Benvin, J. J. Greene, P. O. P. Ts'o, and C. Baglioni.** 1980. Activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells by 2′-O-methylated poly (inosinic acid) · poly (cytidylic acid). J. Biol. Chem. 255:6403–6407.
46. **Ochoa, S.** 1983. Regulation of protein synthesis initiation in eukaryotes. Arch. Biochem. Biophys. 223:325–349.
47. **O'Malley, R. P., R. F. Duncan, J. W. B. Hershey, and M. B. Mathews.** 1989. Modification of protein synthesis initiation factors and shut-off of host protein synthesis in adenovirus-infected cells. Virology 168:112–118.
48. **O'Malley, R. P., T. M. Mariano, J. Siekierka, and M. B. Mathews.** 1986. A mechanism for the control of protein synthesis by adenovirus VA RNA_I. Cell 44:391–400.
49. **O'Malley, R. P., T. M. Mariano, J. Siekierka, W. C. Merrick, P. A. Reichel, and M. B. Mathews.** 1986. The control of protein synthesis by adenovirus VA RNA. Cancer Cells 4:291–301.
50. **Patel, R. C., and G. C. Sen.** 1992. Identification of the double-stranded RNA-binding domain of the human interferon-inducible protein kinase. J. Biol. Chem. 267:7671–7676.
51. **Pestka, S., J. A. Langer, K. C. Zoon, and C. E. Samuel.** 1987. Interferons and their actions. Annu. Rev. Biochem. 56:727–777.
52. **Petryshyn, R., J.-J. Chen, and I. M. London.** 1984. Growth-related expression of a double-stranded RNA-dependent protein kinase in 3T3 cells. J. Biol. Chem. 259:14736–14742.
53. **Rice, A. R., M. Kostura, and M. B. Mathews.** 1989. Identification of a 90 KD polypeptide which associates with adenovirus VA RNA_I and is phosphorylated by the double-stranded RNA dependent protein kinase. J. Biol. Chem. 264:20632–20637.
54. **Samuel, C. E.** 1991. Antiviral actions of interferon: interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology 183:1–11.
55. **Schmedt, C., S. R. Green, and M. B. Mathews.** Unpublished data.
56. **Schneider, R. J., and T. Shenk.** 1987. Impact of virus infection on host cell protein synthesis. Annu. Rev. Biochem. 56:317–332.
57. **Schwaitz, H., and J. P. Ebel.** 1971. A study of the mechanism of action of E. coli ribonuclease III. Biochimie 53:585–593.
58. **Sen, G. C., H. Taira, and P. Lengyel.** 1978. Interferon, double-stranded RNA, and protein phosphorylation. J. Biol. Chem. 253:5915–5921.
59. **Sonenberg, N.** 1990. Measures and countermeasures in the modulation of initiation factor activities by viruses. New Biol. 2:402–409.
60. **Svensson, C., and G. Akusjärvi.** 1985. Adenovirus VA RNA_I mediates a translational stimulation which is not restricted to the viral mRNAs. EMBO J. 4:957–964.
61. **Tiwari, R. K., J. Kusari, R. Kumar, and G. C. Sen.** 1988. Gene induction by interferons and double-stranded RNA: selective inhibition by 2-aminopurine. Mol. Cell. Biol. 8:4289–4294.
62. **Torrence, P. F., M. I. Johnston, D. A. Epstein, H. Jacobsen, and R. M. Friedman.** 1981. Activation of human and mouse 2-5A synthetases and mouse protein P_1 kinase by nucleic acids. FEBS Lett. 130:291–296.
63. **Zilberstein, A., A. Kimchi, A. Schmidt, and M. Revel.** 1978. Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. Proc. Natl. Acad. Sci. USA 75:4734–4738.
64. **Zinn, K., A. Keller, L.-A. Whitemore, and T. Maniatis.** 1988. 2-Aminopurine selectively inhibits the induction of β-interferon, *c-fos*, and *c-myc* gene expression. Science 240:210–213.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 254, No. 20, Issue of October 25, pp. 10186–10183, 1979
Printed in U.S.A.

Structural Requirements of Double-stranded RNA for the Activation of 2′,5′-Oligo(A) Polymerase and Protein Kinase of Interferon-treated HeLa Cells*

(Received for publication, May 16, 1979)

Michael A. Minks, Deborah K. West, Susan Benvin, and Corrado Baglioni

From the Department of Biological Sciences and Center for Biological Macromolecules, State University of New York at Albany, Albany, New York 12222

Addition of double-stranded RNA (dsRNA) to extracts of interferon-treated HeLa cells results in the synthesis of 2′,5′-oligo(A) from ATP and in the phosphorylation of a ribosome-associated protein of M_r = 72,000. Previously described assays were used to investigate the structural requirements of dsRNA for the activation of these two enzymatic activities. Poly(CG) with different ratios of C/G was synthesized with polynucleotide phosphorylase. These polynucleotides were either annealed with poly(I) to form mismatched dsRNA or digested with ribonuclease T_1 to produce smaller polynucleotides. Polymers with an average of one mismatch every eight nucleotides failed to activate the 2′,5′-oligo(A) polymerase and protein kinase, whereas polymers with a mismatch every 45 nucleotides were fully active. The polynucleotides obtained by T_1 digestion of poly(CG) were fractionated by gel filtration into discrete size polymers. These sized polynucleotides were annealed with high molecular weight poly(I) and assayed for activation of 2′,5′-oligo(A) polymerase and protein kinase. These enzymes could not be activated by dsRNA containing poly(C) shorter than 30 nucleotides. Maximal activation was obtained with dsRNA containing poly(C) longer than 65 to 80 nucleotides. A similar size requirement for activation was observed with dsRNA formed with poly(A) and poly(U) of known length. These results indicate that a relatively long stretch of base pairs, uninterrupted by either a mismatch or a discontinuity in one of the complementary strands, is required for the activation of the two enzymes studied. These structural characteristics are similar to those previously shown to be required for the induction of interferon by dsRNA.

Natural and synthetic dsRNAs[1] are among the most potent interferon inducers (1). Furthermore, dsRNA is also a potent inhibitor of protein synthesis in extracts of interferon-treated cells (2). This inhibition is due to elevated levels of a protein kinase and an oligonucleotide polymerase which require dsRNA, ATP, and Mg^{2+} for activity (see Ref. 3). Activation of the protein kinase by dsRNA results in the phosphorylation of a polypeptide of about M_r = 70,000 and of the α subunit of initiation factor eIF-2 (3, 4). The oligonucleotide polymerase, designated 2′,5′-oligo(A) polymerase (3), synthesizes from ATP a series of oligonucleotides containing the unusual 2′,5′-phosphodiester linkage (5). The 2′,5′-oligo(A) is not itself inhibitory to protein synthesis, but activates an endonuclease present in both control and interferon-treated cells and inhibition of protein synthesis occurs via mRNA degradation (6, 7).

Relatively little is known about the molecular features of dsRNA which are required for the activation of the protein kinase and 2′,5′-oligo(A) polymerase. RNA/DNA hybrids and the triple-stranded polymer poly(A)·poly(U)·poly(U) do not activate the latter enzyme (8). In contrast, there is vast literature on the molecular features of dsRNA which are relevant for the induction of interferon (1). For example, the presence of mismatched nucleotides in synthetic dsRNA causes a loss of activity (9) and the dsRNA must be longer than approximately 50 base pairs to induce interferon (10).

We report here the results of an investigation of the effect of mismatched nucleotides and of polymer size on the activation of 2′,5′-oligo(A) polymerase and protein kinase by dsRNA. Interestingly, the structural features of dsRNA relevant for the activation of these enzymes are similar to those reported in the literature for the induction of interferon.

EXPERIMENTAL PROCEDURES

Chemicals—Radiochemicals were purchased from New England Nuclear; sized poly(A) and poly(U) from Miles; polynucleotide phosphorylase from P-L Biochemicals; nuclease T_1 from Sigma.

Cells, Extracts, and Assays—HeLa cells grown in suspension culture were treated with 100 reference units/ml of human fibroblast interferon (3×10^5 units/mg) for 17 h prior to harvest. Interferon was obtained from the Interferon Working Group, National Cancer Institute, NIH. Extracts were prepared from these cells as previously described (8). Ribosomes were isolated by centrifugation and resuspended as described (11). Protein kinase activity was determined in assays containing 0.1 mM [γ-^{32}P]ATP (1.3 Ci/mol) and 30 to 40 μg of ribosomes in a final volume of 30 μl. Incubation was at 30°C for 7 min prior to fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). Autoradiographs of the gels were scanned at 560 nm, and the area under the peak corresponding to a polypeptide of M_r = 72,000 was measured as previously described (12). Assays for the synthesis of 2′,5′-oligo(A) contained unless otherwise indicated 5 μl of cell extract (about 10 mg protein/ml), 0.12 M KOAc, 25 mM $Mg(OAc)_2$, 20 mM Hepes/KOH, pH 7.4, 5 mM [^{3}H]ATP (1.6 Ci/mol), 4 mM fructose 1,6-bis-phosphate, 1 mM dithiothreitol, and the indicated amount of dsRNA in a final incubation volume of 25 μl. Incubation was at 30°C for 60 min and reactions were terminated by heating to 95°C for 3 min. The 2′,5′-oligo(A) formed was determined by chromatography on DEAE-cellulose as previously described (8).

Preparation of CG Copolymers, Sized Polynucleotides, and Double-stranded RNA—Ribonucleotide diphosphates were polymerized with *Micrococcus lysodeikticus* polynucleotide phosphorylase (2 mg/ml) at 37°C for 2 h as described (9). Reactions contained CDP and [^{3}H]GDP in molar ratios of 10:1 to 200:1. Polynucleotides were extracted with phenol and dialyzed exhaustively (9). The C and G

* This work was supported by Grants AI-11887 and HL-17710 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: dsRNA, double-stranded RNA; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

content of polynucleotides was calculated from the A_{271}/counts per min ratio of dialyzed material digested overnight with 0.3 N KOH by comparison of this ratio with that of the unpolymerized starting material. Sized polynucleotides were prepared from poly(CG) by 3-h digestion at 37°C with 0.5 to 1 unit of ribonuclease T_1 per A_{271} unit. The digestion products were applied to columns of Sephadex G-200, G-150, or G-50, depending on the expected size of the fragments generated. The size of the polynucleotides in the eluted fractions was estimated from the A_{260}/counts per min ratio. Correction was made for variation of extinction coefficient with chain length by assuming a negligible contribution to absorbance by the G residue present in the polynucleotides and taking absorbance values for oligo(C) from Adler *et al.* (13). Double-stranded RNA was formed by heating equimolar nucleotide amounts of complementary RNA species to 70°C in a buffer containing 0.1 M KOAc and 20 mM Hepes/KOH, pH 7.4, and cooling to 30°C. Poly(I) of $M_r > 100{,}000$ ($s_{20,w} = 9.4$) was used.

RESULTS

Effect of Base Mismatching on the Activation of 2′,5′-Oligo(A) Polymerase and Protein Kinase—Polyribonucleotides containing different ratios of C to G were synthesized with polynucleotide phosphorylase. These polynucleotides were annealed with poly(I) to form dsRNA containing mismatched bases, since G cannot form a normal Watson-Crick base pair with I. The relative mismatching of these dsRNAs is inversely proportional to the C/G ratio. The dsRNAs are designated by the C/G ratio of the poly(CG) strand.

Polymers with a C/G ratio of 7 do not promote synthesis of 2′,5′-oligo(A) or phosphorylation of the M_r = 72,000 polypeptide (Figs. 1, 2, *A* and *D*). Polymers with a C/G ratio of 15 are partially active in both assays and polymers with a ratio of 45 or higher are fully active.

The effect of increasing concentrations of polymer on the synthesis of 2′,5′-oligo(A) was next investigated. Polymers with a C/G ratio of 45 show maximum activity at 5 μg/ml, whereas a polymer with a ratio of 7 shows less than 5% of this activity at 20 μg/ml (data not shown). The activity of polymers of different C/G ratio cannot be explained by differential degradation of these polynucleotides. No degradation to acid-soluble material was detected when polymers of C/G ratio of 7 and 15 were incubated for 90 min under the conditions of our assays (data not shown). The simplest interpretation of the above results is that a minimum length of perfectly matched I·C base pairs is necessary for activation of 2′,5′-oligo(A) polymerase and protein kinase.

Size Requirements for Activation of 2′,5′-Oligo(A) Polymerase and Protein Kinase—Polynucleotides of different C/G ratios were digested with ribonuclease T_1 to yield a series of poly(C) fragments terminating in G (9). These fragments were fractionated by gel filtration and their size determined as described under "Experimental Procedures." In order to generate a full series of sized fragments, polynucleotides with C/G ratios of 200, 45, and 20 were digested and fractionated in this way. Sized poly(C) fragments ranging in average length between 10 and 380 nucleotides were thus obtained. These sized polynucleotides were annealed with equimolar nucleotide amounts of poly(I). The dsRNAs formed are designated as $I_n \cdot C_x$, where x indicates the average number of C residues of a sized polynucleotide terminating in G. The dsRNAs were assayed for activation of 2′,5′-oligo(A) polymerase and protein kinase (Figs. 1 and 2, *B* and *E*). Both enzymes were activated only by dsRNAs containing poly(C) longer than 35 nucleotides, with maximal activity being observed with $I_n \cdot C_{55}$ for synthesis of 2′,5′-oligo(A) and with $I_n \cdot C_{80}$ in the kinase assay.

Partial activation of 2′,5′-oligo(A) polymerase and protein kinase by dsRNAs containing poly(C) 35 to 50 nucleotides long could be due to contamination of the sized polynucleotides with poly(C) of greater length. To test this possibility, we determined the effect of increasing concentrations of dsRNA on the activation of 2′,5′-oligo(A) polymerase (Fig. 3*A*). The polymer $I_n \cdot C_{32}$ was inactive below 10 μg/ml, but at 20 μg/ml had 3% of the activity of $I_n \cdot C_{45}$. We cannot distinguish, however, between a 3% contamination of C_{45} with longer poly(C) and a marginal activation of the enzyme by $I_n \cdot C_{32}$.

In the experiments described above, the 2′,5′-oligo(A) polymerase assay was carried out at 20 mM $Mg(OAc)_2$, which gives optimal synthesis of 2′,5′-oligo(A) with HeLa cell extracts (8). When 2′,5′-oligo(A) synthesis was assayed at the same $Mg(OAc)_2$ used in the kinase assay (2 mM), an increase in the size of dsRNA required for activation was observed. The polymer $I_n \cdot C_{27}$ showed little activity and $I_n \cdot C_{32}$ was

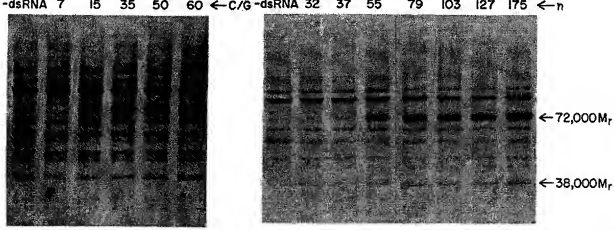


FIG. 1. Phosphorylation of the M_r = 72,000 polypeptide by the protein kinase activated with poly(I)·poly(CG) (*left*) or by poly(I) annealed to sized poly(C) (*right*). Ribosomes from interferon-treated HeLa cells were incubated with or without 0.5 (*left*) or 0.1 (*right*) μg/ml of dsRNA for 7 min at 30°C and analyzed by gel electrophoresis and autoradiography as described under "Experimental Procedures." The tracks show from *left* to *right*: an incubation without added dsRNA; incubations with added poly(I)-poly(CG), with the ratio C/G of the polymer indicated for each track; an incubation without added dsRNA; incubations with added poly(I) annealed to poly(C) of the length indicated for each track (*n*). The position of the M_r = 72,000 and 38,000 polypeptides is indicated on the *right*.

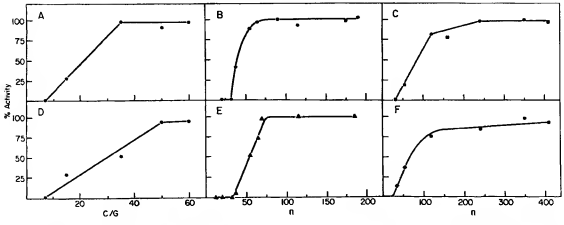


FIG. 2. **Synthesis of 2′,5′-oligo(A) (*A*, *B*, and *C*) and phosphorylation of the M_r = 72,000 polypeptide (*D*, *E*, and *F*) promoted by poly(I)·poly(CG) with different ratios of C/G (*A* and *D*), by poly(I) annealed to sized poly(C) (*B* and *E*), and by sized poly(A) annealed to sized poly(U) (*C* and *F*).** Preparation of polymers and assays for 2′,5′-oligo(A) polymerase and protein kinase are described under "Experimental Procedures." The polymers were tested at 10 μg/ml in the polymerase assay and at 0.5 (*D*) or 0.1 (*E* and *F*) μg/ml in the kinase assay. The activity is expressed as a percentage of that obtained with poly(I)·poly(C). With this polymer, 33 nmol of ATP were converted to 2′,5′-oligo(A) from an input of 125 nmol of ATP per reaction. In the kinase assay, phosphorylation was determined by scanning autoradiographs of gels, like those shown in Fig. 1, and measuring the area under the M_r = 72,000 band. Phosphorylation observed with different polymers is shown as a percentage of that obtained with poly(I)·poly(C). On the *abscissa* is indicated the C/G ratio of the polymers tested (*left panels*), the length in nucleotides of the poly(C) annealed to poly(I) (*middle panels*), and the length of the shorter polynucleotide used to form dsRNA with sized poly(A) and poly(U) (*right panels*). Different symbols designate in these latter panels the longer complementary polynucleotides annealed: (●) A_{100}, (◆) U_{120}, and (■) U_{500}.

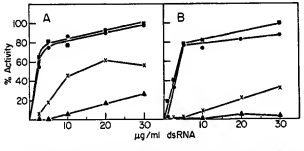


FIG. 3. **Activation of 2′,5′-oligo(A) polymerase by different concentrations of poly(I) annealed to sized poly(C) with (*A*) 20 mM and (*B*) 2 mM $Mg(OAc)_2$.** Increasing concentration of poly(I)·C_{12} (▲—▲), poly(I)·C_{27} (×—×), poly(I)·C_{45} (●—●), and poly(I)·C_{260} (■—■) were added to standard incubation mixtures. With 30 μg/ml of poly(I)·poly(C) and 20 mM $Mg(OAc)_2$, 40 nmol of ATP were converted to 2′,5′-oligo(A) from an input of 125 nmol, whereas 20 nmol were converted with 2 mM $Mg(OAc)_2$. Each reaction contained also 5 mM $Mg(OAc)_2$ added in equimolar amount with ATP (see "Experimental Procedures"). The synthesis of 2′,5′-oligo(A) is expressed as a percentage of that obtained with 30 μg/ml of poly(I)·poly(C).

practically inactive even at 30 μg/ml (Fig. 3*B*), whereas $I_n \cdot C_{51}$ remained fully active.

The failure of dsRNAs containing short poly(C) to activate either the 2′,5′-oligo(A) polymerase or the protein kinase may be due to low affinity of the enzymes for these polymers. Alternatively, these dsRNAs may bind the enzymes but may not function as activators. This dsRNA/enzyme interaction was studied by competition experiments, in which an active dsRNA was assayed in the presence of increasing amounts of inactive dsRNA. No competition between 2.5 μg/ml of active dsRNA and 30 μg/ml of inactive dsRNA was detected in the 2′,5′-oligo(A) polymerase assays (data not shown). The results of experiments testing competition in the protein kinase assay were more difficult to interpret. Activation of the kinase occurs only within a range of dsRNA concentrations, and high dsRNA levels are inhibitory (14). Addition of a 10-fold excess of $I_n \cdot C_{30}$ had no effect on kinase activation by 0.1 μg/ml of $I_n \cdot C_{120}$, whereas a 100-fold excess significantly impaired kinase activation. The concentration of dsRNA was raised in this case to 10 μg/ml. Both short and long polymers were inactive at this dsRNA concentration (Ref. 11 and data not shown). It seems possible, therefore, that inactive dsRNAs may prevent activation of the kinase by raising the dsRNA concentration to the inhibitory range.

Sized poly(A) and poly(U) were similarly tested upon annealing with equimolar nucleotide amounts of complementary strands. It was therefore possible to construct dsRNAs with two polynucleotides of known length. The dsRNAs containing A_{33} were inactive in the 2′,5′-oligo(A) polymerase assay and only slightly active in the kinase assay (Fig. 2, *C* and *F*). The dsRNAs containing A_{54} were partially active in both assays, and those containing U_{120} annealed to longer poly(A) were fully active. The effect of the complementary chain length on the activation of 2′,5′-oligo(A) polymerase was investigated in a systematic way (Table I). The activity of the dsRNAs was found to be dependent mainly on the length of the shorter polynucleotide. The length of the complementary polynucleotide is also important, however, as shown by the higher activity of A_{54} annealed with poly(U) of increasing length. A

TABLE I

Effect of varying poly(A) and poly(U) size on the synthesis of 2′,5′-oligo(A)

Polynucleotide[a] (average size)		2′,5′-Oligo(A) synthesized
A	U	
		nmol adenosine polymerized
33	120	0
54	120	0.7
54	240	1.4
54	500	6.3
160	120	14.3
160	350	19.1
160	500	25.5
410	240	32.5
410	500	31.7

[a] Equimolar nucleotide amounts of poly(A) and poly(U) were annealed and assayed at 20 μg/ml as described under "Experimental Procedures." Each reaction contained 125 nmol of [^{3}H]ATP.

possible explanation for this effect is that annealing of two relatively short polymers is likely to result in more discontinuities in complementary strands than when a shorter polymer is annealed with a longer one. More double-stranded sequences of the minimal length necessary for enzyme activation will be formed in this latter case.

DISCUSSION

We have studied the activation of two dsRNA-dependent enzymatic activities by preparations of poly(I)·poly(CG) containing different proportions of G residues (mismatched dsRNA). The results obtained clearly indicate that a minimum length of perfectly matched base pairs is necessary for the activation of these enzymes. This length can be roughly estimated by calculating the average frequency of C runs of different length in polymers with variable degree of mismatching. If a random distribution of C and G residues is assumed to occur in these polymers, the probability of finding a C run of length n is then given by $(C/(C+G))^n$ (where $(C/(C+G))$ is the relative proportion of C in the mismatched strand). Runs of 35 or more C's are 40-fold more frequent in a polymer with a C/G ratio of 35 than in a polymer with a ratio of 7. Experimentally, the former polymer was found to be at least 20-fold more active than the latter polymer in promoting synthesis of 2',5'-oligo(A).

A direct estimate of the dsRNA size requirement for activation of these enzymes was obtained by forming polymers with a high molecular weight poly(I) strand annealed to poly(C) of different length. Only dsRNA containing poly(C) longer than 65 to 80 nucleotides was fully active. These results were confirmed with poly(A) and poly(U) of known size annealed in different combinations. The structural requirements of dsRNA for activation of the 2',5'-oligo(A) polymerase and protein kinase are therefore similar. There are, however, some differences between these two enzymes. The 2',5'-oligo(A) polymerase is fully activated by poly(I)·poly(CG) with fewer mismatches than the kinase, and dsRNA of greater size is required for activation of this latter enzyme. These differences may in part be explained by the different Mg^{2+} concentration in the assays, since slightly larger dsRNA was required for maximal activation of 2',5'-oligo(A) polymerase at lower Mg^{2+} concentration. The activation of these two enzymes differs in another way: the kinase cannot be activated in the presence of high concentrations of dsRNA (14), whereas the 2',5'-oligo(A) polymerase is activated more effectively by high concentrations of dsRNA (8).

There are similarities between the structural requirements of dsRNA for interferon induction and those for activation of 2',5'-oligo(A) polymerase and protein kinase. These similarities have been previously noticed in studies assaying the inhibition of protein synthesis by dsRNA in reticulocyte lysates (15) and extracts of interferon-treated L cells (16), presumably due to the combined action of the protein kinase and 2',5'-oligo(A) polymerase/endonuclease system. A threshold molecular size of dsRNA corresponding to approximately 50 base pairs determines the interferon inducing activity of dsRNA (10). A similar size requirement is observed for the inhibition of protein synthesis in reticulocyte lysates by dsRNA (14). These observations agree with our findings that about 40 to 60 base pairs of dsRNA are required for the activation of 2',5'-oligo(A) polymerase and protein kinase under different assay conditions. Similarly, the ability of dsRNA to induce interferon decreases with increasing content of mismatched bases (9, 17).

Our results are directly comparable to those obtained by Carter *et al.* (9) with poly(I)·poly(C_{20}G). This polymer can partially activate the 2',5'-oligo(A) polymerase and protein kinase and has an intermediate interferon-inducing activity. Further work on the interferon-inducing activity of the other polymers used in our studies could possibly provide additional evidence for this correlation.

Some differences between the interferon-inducing activity and the inhibitory effect on protein synthesis of natural and synthetic dsRNA have been previously described (15, 16). Certain synthetic dsRNAs containing modified nucleotides are extremely efficient interferon inducers but do not significantly inhibit protein synthesis in reticulocyte lysates (15) or in extracts of interferon-treated L cells (16). Assays of these dsRNAs for activation of the 2',5'-oligo(A) polymerase and protein kinase will establish how closely the interferon-inducing activity of dsRNA is correlated with its ability to activate these enzymes. It is tempting to speculate that a dsRNA-dependent interferon-induced enzyme is part of the cellular recognition system for dsRNA. Both 2',5'-oligo(A) polymerase and protein kinase are present at a basal level in all mammalian and avian cells studied thus far (3). A dsRNA formed by a replicating virus or administered to cells may interact with these enzymes, which are known to bind dsRNA (18), and activate synthesis of 2',5'-oligo(A). This compound may in turn have some biological role in the induction of interferon synthesis. Further studies are necessary to provide some experimental support for this working hypothesis.

REFERENCES

1. Torrence, P. F., and De Clercq, E. (1977) *Pharmacol. Ther. Part A* **2**, 1–88
2. Kerr, I. M., Brown, R. E., and Ball, L. A. (1974) *Nature* **250**, 57–59
3. Baglioni, C. (1979) *Cell* **17**, 255–264
4. Sen, G. C., Taira, H., and Lengyel, P. (1978) *J. Biol. Chem.* **253**, 5915–5921
5. Kerr, I. M., and Brown, R. E. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 256–260
6. Baglioni, C., Minks, M. A., and Maroney, P. A. (1978) *Nature* **273**, 684–687
7. Clemens, M. J., and Williams, B. R. G. (1978) *Cell* **13**, 565–572
8. Minks, M. A., Benvin, S., Maroney, P. A., and Baglioni, C. (1979) *J. Biol. Chem.* **254**, 5058–5064
9. Carter, W. A., Pitha, P. M., Marshall, L. W., Tazawa, I., Tazawa, S., and Ts'o, P. O. P. (1972) *J. Mol. Biol.* **70**, 567–587
10. DeClercq, E. (1974) *Top. Curr. Chem.* **52**, 173–208
11. Lenz, J. R., and Baglioni, C. (1978) *J. Biol. Chem.* **253**, 4219–4223
12. West, D. K., and Baglioni, C. (1979) *Eur. J. Biochem.*, in press
13. Adler, A., Grossman, L., and Fosman, G. D. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **57**, 423–430
14. Hunter, T., Hunt, T., Jackson, R. J., and Robertson, H. D. (1975) *J. Biol. Chem.* **250**, 409–417
15. Content, J., Lebleu, B., and De Clercq, E. (1978) *Biochemistry* **17**, 88–93
16. Torrence, P. F., and Friedman, R. M. (1979) *J. Biol. Chem.* **254**, 1259–1267
17. Matsuda, S., Kida, M., Shirafugi, H., Yoneda, M., and Yaoi, H. (1971) *Arch. Gesamte Virusforsch.* **34**, 105–118
18. Hovanessian, A. G., and Kerr, I. M. (1979) *Eur. J. Biochem.* **93**, 515–526